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Inorganic Sulfate: An Important Dietary Constituent for the Monogastric Animal

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John T. Smith

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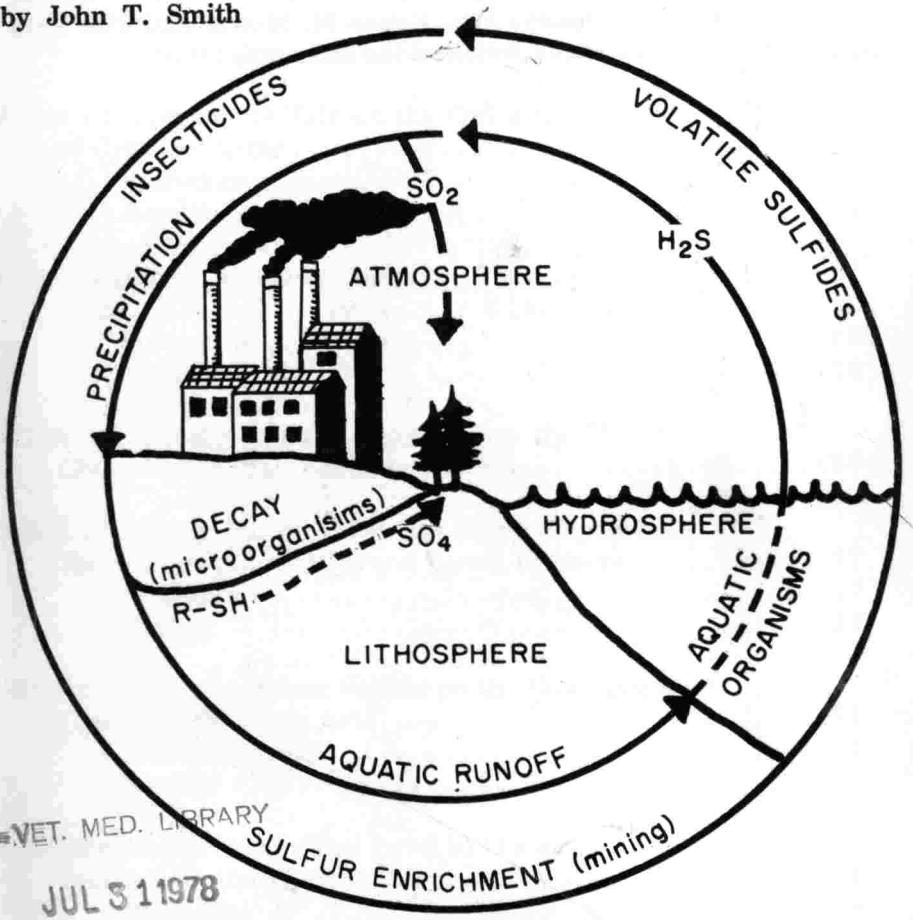

The University of Tennessee
Agricultural Experiment Station
D. M. Gossett, Dean
Knoxville

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INORGANIC SULFATE: AN IMPORTANT DIETARY CONSTITUENT FOR THE MONOGASTRIC ANIMAL

by John T. Smith*

INTRODUCTION:

Much of the literature related to sulfur nutrition and metabolism either ignores or dismisses the importance of inorganic sulfate to the nutrition of a monogastric animal. Consideration of the information presented in Figures 1 and 2 will help to explain why most investigators have ignored the importance of inorganic sulfate. The compounds listed in Figure 1 are sulfur-containing compounds important to body functions. The letters in parenthesis refer to the oxidation state of the sulfur in these compounds. Of the 15 compounds listed in this figure, only five (1/3) of them contain sulfur at the oxidation state of sulfate.

Selected schemes representing the metabolism of the sulfur-containing amino acids are shown in Figure 2. Notice that the sulfur atom from either methionine or cysteine may eventually be oxidized to yield endogenous inorganic sulfate. These two observations then have prompted nutritionists to argue that dietary inorganic sulfate is of little importance based on the relatively few compounds in the body that contain sulfur as sulfate and the ease of production of endogenous inorganic sulfate from the sulfur-containing amino acids.

The discussion which follows represents a complication and interpretation of data collected over a period of several years. These data show that dietary inorganic sulfate may have a sparing effect on the sulfur-containing amino acids and that unless metabolic alterations are to occur, the diet of a monogastric animal must contain an optimal level of inorganic sulfate.

It is difficult to assess the relative importance of sparing the sulfur-containing amino acids and avoiding metabolic alterations

*Professor, Department of Food Science, Nutrition, and Food Systems Administration.

Figure 1. Comparison of the oxidation levels of sulfur compounds important to man, animals and plants.

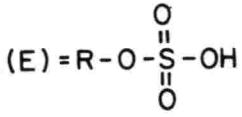
A. Hormones
   1. Insulin (H)
   2. Antidiuretic (H)
   3. Gastrin II (E)

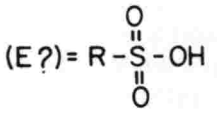
B. Regulatory Compounds
   1. Sulfhydryl Enzymes (H)
   2. Coenzyme A (H)
   3. Acyl Carrier Protein (H)

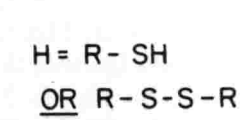
C. Structural Components
   1. Mucopolysaccharides (E)
   2. Sulfolipids (E)
   3. Methionine and Cysteine (H)

D. Other
   1. Heparin (E)
   2. Bile Salts (E)
   3. Glutathione (H)
   4. Mercapturic Acids (H)

E. Vitamins, Growth Factors
   1. Biotin (H)
   2. Thiamin (H)
   3. Lipoic Acid (H)







since they are interrelated; however, the impending shortage of protein supply in the world is of paramount importance to everyone. For example, Dr. W. H. Allaway (1) in the keynote talk at a Symposium entitled, "Sulfur in Nutrition," stated that there is now a shortage in the sulfur-containing amino acids for human and animal nutrition and that it will probably get worse. His argument is that the sulfur cycle is threatened by man's pressure on agriculture for increased production, the use of purified fertilizers, and the restrictions on sulfur emission into the atmosphere.

Since all of the experimental data to be reported were obtained by feeding diets of different sulfur content to rats, a general description of diets will be presented, and then only specialized techniques will be discussed as each experiment is presented. The composition of the diets, including the special salt mixture used to achieve diets with different inorganic sulfate contents, is shown in Table 1. A 15% casein diet was selected as our reference diet, since it supplies all of the other amino acids at a level sufficient for optimum growth but is limiting in the sulfur-containing amino acids (2). Both methionine and cysteine are shown in this table as added dietary constituents. However, any one experiment was usually conducted with only one of the sulfur-containing amino acids as supplement.

Figure 2. Schematic representation of the metabolic pathways leading to the formation of taurine and sulfate from the sulfur-containing amino acids, methionine, cysteine, cystine.

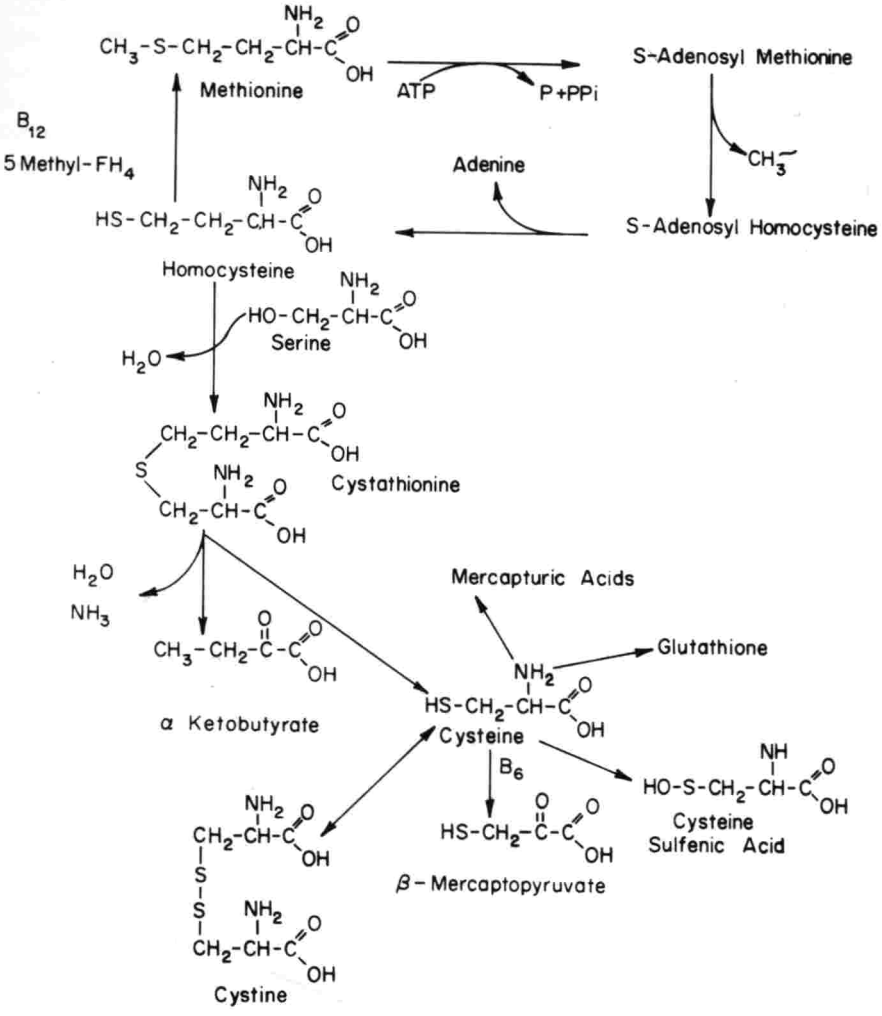


Figure 2. (Continued).

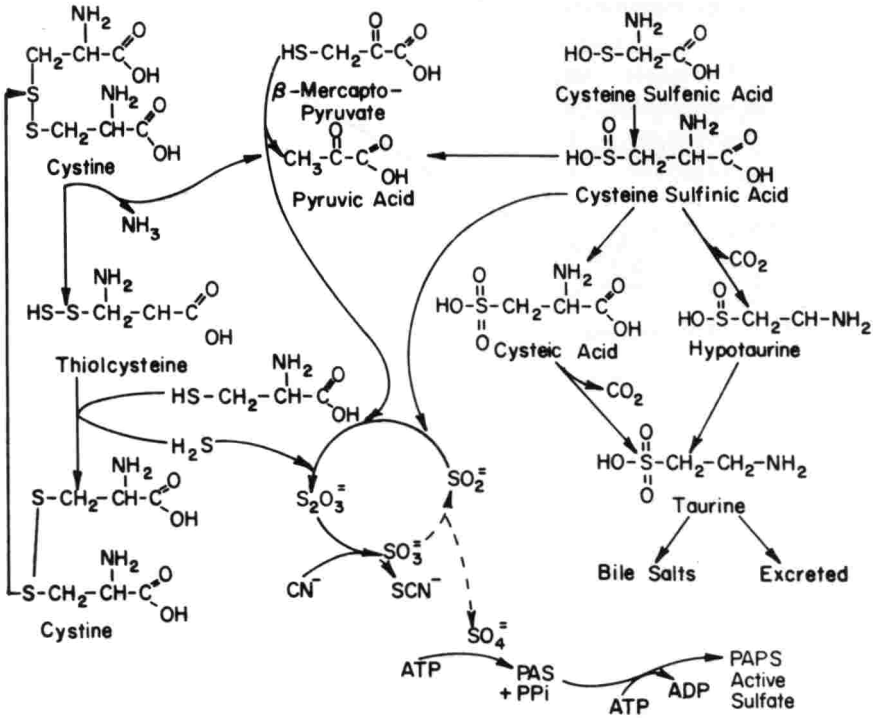


Table 1. Composition of diets

Component	0.0002% SO_4	0.02% SO_4	0.1% SO_4	0.42% SO_4
Casein	15.00	15.00	15.00	15.00
Sucrose	30.00	30.00	30.00	30.00
Cornstarch	30.00	30.00	30.00	30.00
Cod-liver oil	2.00	2.00	2.00	2.00
Vegetable shortening[1]	6.00	6.00	6.00	6.00
Vitamin mixture[2]	2.00	2.00	2.00	2.00
Basal salt mixture[3]	1.34	1.34	1.34	1.34
$CaSO_4$	0.00	0.04	0.18	0.75
$CaCo_3$	1.34	1.32	1.23	0.91
Methionine	0.0-0.67	0.0-0.62	0.0-0.57	0.0
Cysteine[4]	0.0-0.53	0.0-00.5	0.0-00.4	0.0
Non-nutritive bulk[5]	11.66	11.68	11.75	12.00

[1]Crisco, Proctor and Gamble.

[2]Nutritional Biochemicals Corp., Cleveland, OH 44128, Vitamin Diet Fortification Mixture formulated to supply the following amounts of vitamins (g/kg Vitamin premix) Thiamin hydrochloride 1.0, riboflavin 1.0, niacin 4.5, p-aminobenzoic acid 5.0, calcium pantothenate 3.0, pyridoxine hydrochloride 1.0, ascorbic acid 45, inositol 510, choline chloride 75, menadione 2.25, biotin 0.020, folic acid 0.090, vitamin B_{12} 0.00135, α-tocophecol 5.0, vitamin A 9×10^5 units, vitamin D 1×10^5 units, and enough glucose to make 1000 g.

[3]Formulated to supply the following amounts of minerals (g/450 g salt mixture): magnesium carbonate 30.6, sodium chloride 69.0 potassium chloride 112, potassium monobasic phosphate 212, ferric phosphate 20.5, potassium iodide 0.08, sodium fluoride 0.1, manganese chloride 0.4, aluminum potassium sulfate 0.1, and cupric acetate 0.72.

[4]Cysteine-free base.

[5]Alphacel Nutritional Biochemical Corp., Cleveland, Ohio 44128.

Comparison of the Utilization of Organic and Inorganic Sulfur in the Rat

Previous work in this laboratory has shown that the response of rats to avitaminosis E is related not only to the total sulfur in the diet but also to the ratio of inorganic to neutral sulfur (3). These data demonstrate that fortified liver homogenates from vitamin E-deficient rats were less effective in converting the sulfur of cysteine-^{35}S to $^{35}SO_4$ than those from their E-sufficient litter mates. These observations would indicate that the rat was using inorganic sulfate from the diet. However, as stated in the introduction, previous publications have suggested that the sulfate from inorganic sources is poorly utilized by animals (4).

Therefore, since our data indicated that inorganic sulfate was used by the rat and the literature did not support this finding, an experiment was designed to compare the utilization of inorganic and organic sulfur. Cartilage mucopolysaccharides which are comparatively easy to isolate and exhibit measurable sulfur uptake were chosen as the natural product to isolate and analyze to assay sulfur utilization. Since ^{35}S was used both in testing inorganic and organic sulfate utilization, the ^{35}S activity in the cartilage mucopolysaccharides should reflect sulfate utilization.

PROCEDURE:

Twenty adult, female, albino rats of the Wistar strain were used, five rats per treatment group, to test the utilization of $Ca^{35}SO_4$ and methionine ^{35}S. The rats were fed the 0.0002% sulfate diet described in Table 1 with 0.67% methionine (diet 4), the 0.1% sulfate diet with 0.57% methionine (diet 2 and 3), and the 0.42% sulfate diet (diet 1) with no added sulfur-containing amino acids. The rats were fed these diets for a period of 17 days. The first 7 days the diet was non-radioactive. During the last 10 days of the period, the $^{35}CaSO_4$ was supplied in diets 1 and 3 and ^{35}S methionine was supplied in diets 2 and 4. During the radioactive period, fecal collections were made daily. At the end of each dietary period the animals were sacrificed by a blow to the head followed by decapitation. Rib cartilage and blood samples were saved from each animal. The rib cartilage was frozen until mucopolysaccharides could be extracted from them and analyzed by Bostrom's method (5). The activity of ^{35}S in samples of feces, blood, and mucopolysaccharides was determined by the method of Katz and Golden (6).

RESULTS:

Comparison of the data, which are shown in Table 2 for diets 2 and 3 which are identical except for the source of ^{35}S, demonstrated that the sulfate ion from inorganic sources contributes as much to the metabolic pool as does endogenous sulfate oxidized from organic sources; the counts per min per m mole of sulfate in the mucopolysaccharide fraction is not significantly different between the rats fed diet 2—in which the ^{35}S methionine was radioactive—and diet 3 in which $Ca^{35}SO_4$ was radioactive. These data also show an attempt by the rat to adapt to a diet low in sulfate, since methionine is absorbed better from the diet containing no inorganic sulfate (diet 1) than from the diet containing 0.1% of sulfate (diet 2), and since the specific activity of the fecal sulfur as sulfate is higher in diet 2 than in diet 1. These data assume increased significance when the lower inorganic sulfur intake is considered. Based on the lower sulfur intake, there should have been less sulfur in the feces; therefore a decrease in specific activity really indicates an increase in absorption

Table 2. Distribution of radioactivity in the Feces, blood, and mucopolysaccharides of rats[1] fed diets containing 35 S-methionine and $Ca^{35}SO_4$

Diet no.	Percent dietary sulfur (as sulfate)		Counts/min/m mole $SO_4^=$ as a percent of total ingested activity		
	Inorganic	Neutral	Feces	Blood	Mucopolysaccharides
1	0.0002	0.67 (^{35}S-meth)	5.06	2.73	0.39
2	0.10	0.57 (^{35}S-meth)	6.35	4.09	0.64
3	0.10 ($Ca^{35}SO_4$)	0.57	8.24	0.15	0.56
4	0.42 ($Ca^{35}SO_4$)	0.25	6.15	0.14	0.78

[1]Six adult female rats in each group.

of methionine with a decrease in inorganic sulfate in the diet.

A comparison of the uptake of ^{35}S by the cartilage mucopolysaccharides in rats fed diet 1 compared to diet 4 also show a better utilization of sulfate from a diet high in inorganic sulfate than one high in neutral sulfur. The low uptake of sulfate by the rib cartilage mucopolysaccharides of those rats fed the diet low in inorganic sulfate (diet 1) indicates that even 1.04% of methionine in the diet—an excess of 0.44% over the 0.6% which is the recommended (2) level for optimum growth by rats—is not sufficient to supply the endogenous sulfate provided by a diet containing 0.1% of sulfate, diets 2 and 3. However, on a molar basis, 0.15% of methionine could supply sulfate equivalent to 0.1%. Therefore, since 0.4% of methionine did not supply enough sulfate, these data give the first indication that if metabolic alterations are to be avoided, inorganic sulfate must be included in the diet.

Routine handling of the rats used in these experiments suggested that there was a decrease in the integrity of the skin of rats fed diets low in inorganic sulfate. This observation led to the following series of experiments with respect to the effect of dietary sulfur on the collagen of skin and aorta.

Collagen Content of Skin and Aorta and Breaking Strength of Aorta

PROCEDURE:

Twenty male and 10 female, Wistar rats from the stock colony of the Nutrition Department, University of Tennessee, were used as test animals. They were fed the 0.0002% and 0.02% sulfate diets described in Table 1 from weaning until they were adults (200 g). Collagen was extracted and fractionated from skin and aortae according to the method of Martin et al. (7). Hydroxproline content of the

collagen fraction was determined by the method of Martin and Axelrod (8).

Breaking strength of the aortae was determined as described below. The experimental animal was placed under anesthesis with ether, decapitated, and bled thoroughly. The heart and several centimeters of aorta were removed and promptly placed in approximately 25 ml of cold (4°) Ringers solution. The aorta was freed from surrounding tissue by blunt dissection, and the heart cut away from the aorta just distal to the arch. This 2.5-3.0 cm segment of the aorta was then promptly measured, blotted, and weighted on a Mettler model B_1 balance and immediately returned to cold Ringers solution. The aorta segment was placed between the rubber-covered jaws of two hemostats so that a uniform section (1 cm long) was broken. Water was added at a constant rate to a small polyethylene bottle suspended by wires from the lower hemostat. The weight required to separate the aorta was determined by weighing the water bottle and hemostat. In order to compensate for differences in thickness of the aortic walls, the breaking strength was calculated as the weight required to break a segment 1 cm long normalized to 1 g.

RESULTS:

The data which are shown in Table 3 show a comparison of the collagen fractions from the skin of rats fed the normal (0.02%) and the low (0.0002%) sulfate diets. These data show that both the total collagen per 100 g of wet skin and the neutral salt soluble collagen per 100 g of collagen are significantly less in those rats fed the diet low in sulfate.

Table 3. Comparison of collagen fractions from skins of rats fed normal sulfate and low sulfate diets

	Collagen[1]			
		Soluble		
Dietary sulfate	g/100g skin	Neutral salt	Acid collagen	total
Low (0.0002%)	24.1	6.0[2]	28.7	34.7
Normal (0.02%)	36.8	10.5	32.0	42.5

[1]Hydroxyproline x 7.46.

[2]Five animals per group.

Since these data demonstrate a relationship between dietary sulfate and the synthesis and total content of collagen in the skin of rats, the aortae of these rats were also evaluated for collagen content and breaking strength. The data obtained from this evaluation presented in Table 4 show a significant decrease in both the collagen content and breaking strength of the aortae from both male and female rats fed diets low in organic sulfate. Although the data which

Table 4. Collagen content and breaking strength of normalized aortic segments from rats fed diets with low and normal sulfate content

	Sulfate level	
	Normal	Low
Collagen MG		
Male	334	178
Female	304	179
Breaking strength (2g/Mg collagen)		
Male	77	59
Female	87	48

are presented in Table 2 indicate a metabolic alteration in methionine metabolism by rats fed diets low in sulfate, these data (Tables 3 and 4) were the first that we had collected showing a metabolic alteration in rats fed diets low in sulfate. And in the latter case an impairment of arterial integrity (Table 4), a metabolic alteration with possible physiological implications, was apparent. The relationship between arterial integrity and the level of dietary inorganic sulfate implied by these data suggested further investigation into the metabolic effects of dietary inorganic sulfate.

Effect of Inorganic Sulfate on the Oxidation of Cysteine Sulfur

It therefore seemed appropriate to design an experiment to determine if a relationship between inorganic sulfate and the oxidation of cysteine sulfur could be demonstrated **in vitro.**

PROCEDURE:

Adult male Wistar rats of the University of Tennessee, Nutrition Department colony were used as tissue donors. The animals were stunned by a blow on the head, decapitated, and bled thoroughly. The liver was then removed to a tared beaker containing a cold 0.067 M solution of phosphate buffered at pH 7.4. A 20% liver homogenate was made with this buffer using a Thomas motor-driven homogenizer. The oxidation of cystine sulfur to sulfate was followed by the method of Patrick (9). Experimental details are given with Table 5. After adding the trichloracetic acid, the protein precipitates were removed by filtration and washed. The filtrates were concentrated and acidified with a few drops of concentrated HCl. ^{35}S-sulfate was precipitated and counted according to the method of Katz and Golden (6). Nitrogen content of the liver homogenate was determined by the micro-Kjeldahl method (10).

Table 5. Effect of sulfate addition on the oxidation of cysteine sulfur to sulfate[1]

μM sulfate in 5 ml incubation mixture	μM sulfate formed per Mg nitrogen	Per cent inhibition
None		
3	0.80[2]	0.0
6	0.79	0.8
15	0.72	10.8
	0.64	20.0
Boiled Enzyme	0.02	98.0

[1]The basic incubation mixture contained 0.5 ml of a 20% whole liver homogenate in 0.067M PO_4 buffer pH 7.4. 125μ moles ^{35}S-cysteine-HCl in buffer: 100μ moles of $MgCl_2$ 10μ moles ATP in buffer, 0.18μ moles NAD^+ in buffer and sufficient buffer to make 5 ml. The samples were incubated in air at 37° for 120 mon. The reaction was stopped by adding 5 ml of 10% trichloroacetic acid.

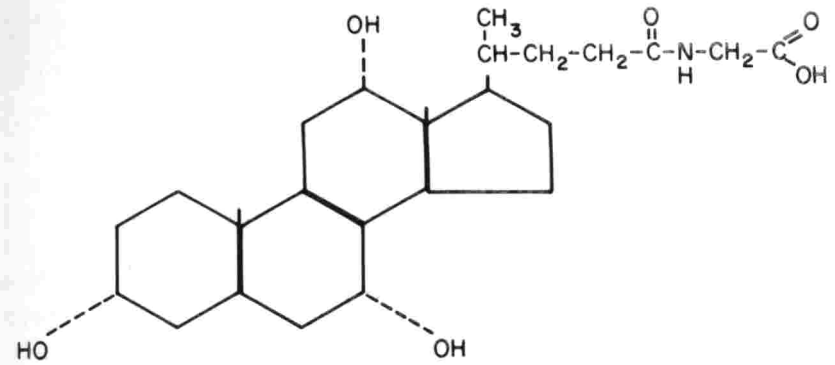
[2]Values are averages of five determinations.

RESULTS:

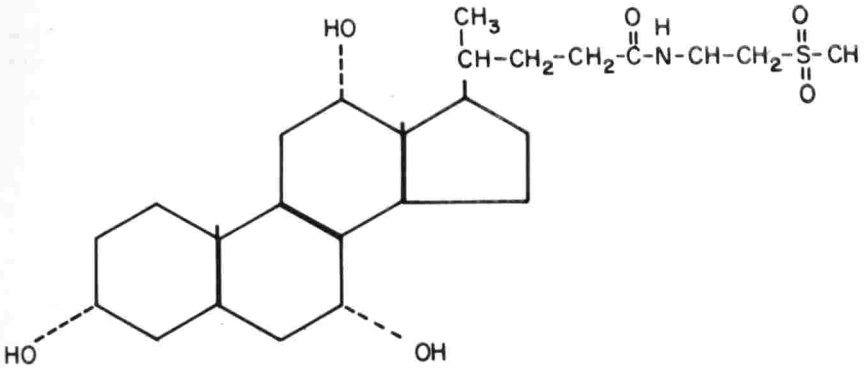
The data shown in Table 5 confirm **in vitro** the suggested relationship between dietary inorganic sulfate and the oxidation of cysteine sulfur to sulfate suggested by the **in vivo** data presented previously. As suggested by these data, inorganic sulfate added to an incubation mixture can reduce the oxidation of cysteine sulfur to sulfate. If this same relationship holds **in vivo**, it allows an attractive explanation for the observation, reported in Table 1: if the inorganic sulfate level of a diet is low, then the utilization of the sulfur-containing amino acids is impaired to the extent that a 3-fold theoretical excess of methionine will not supply enough endogenous sulfate to satisfy the body's needs.

Consideration of Figure 2 shows that cysteine sulfinic acid could be a branch point in the oxidation of cysteine sulfur. Cysteine sulfinic acid may be deaminated to yield pyruvic acid, ammonia, and sulfate, or its sulfur moiety may be oxidized to cysteic acid, or it may be decarboxylated to yield hypotaurine. Either of the latter reactions—the oxidation to cysteic acid or the decarboxylation to hypotaurine—will ultimately yield taurine. If one assumes that low levels of inorganic sulfate stimulate the oxidation of cysteine to cysteine sulfinic acid and that the activity of the transaminase, cysteinesulfinic acid oxidase, and decarboxylase remains constant, then and increase in cysteinesulfinic acid would result in an increase in taurine synthesis at the expense of increased sulfate synthesis. Since tissue taurine levels are somewhat constant (11), the increased endogenous taurine would become a urinary excretory product and represent a wasteful utilization of the sulfur-containing amino acids

Figure 3. Glycocholic: taurocholic ratio G:T ratio



GLYCOCHOLIC ACID



TAUROCHOLIC ACID

by rats fed diets low in inorganic sulfate as suggested by the data in Table 1. Therefore the following experiment was designed to check this explanation for the apparent wasteful metabolism of the sulfur-containing amino acids by rats fed diets low in inorganic sulfate.

Effect of Dietary Inorganic Sulfate on Taurine Excretion

PROCEDURE:

Adult Sprague Dawley rats were fed the 0.0002%, the 0.1%, and the 0.42% sulfate diets as described in Table 1. Cysteine supplementation of each dietary treatment is indicated in Table 6. Notice

Table 6. ^{35}S-cysteine sulfur excreted as ^{35}S-taurine sulfur in a 24-hour urinary collection[1]

Diet no.	Percent dietary sulfate	Cysteine added g/100 g	Total taurine counts/min/24 hr x 10^{-3}
1	0.0002	0.53	3.3
2	0.0002	0.40	2.9
3	0.10	0.40	1.4
4	0.42	0.00	0.6
5	0.42	0.40	1.9

[1]Values represent averages of 10 values obtained by analysis of two 24-hour urine samples from each of five adult rats fed the test diets for 17 days.

that two dietary treatments are indicated by the levels of cysteine supplementation: one in which the total sulfur as sulfate is kept constant (diets 1, 3, and 4), and the other in which the cysteine supplementation is kept constant (diets 2, 3, and 5). Non-radioactive test diets were fed for 10 days and then radioactive diets, prepared by adding 0.05% of ^{35}S-cysteine (2.5 x 10^5 cpm/mg) to each diet, were fed for an additional 7-day period. During the last 2 days, the rats were placed in metabolism cages and two separate 24-hour-urine collections were made. The urine collections were stored at -20° until they could be processed for the isolation of ^{35}S-taurine. ^{35}S-taurine was isolated from the urine by the method of Sorbo (12) after it had been deproteinized according to the method of Somogyi (13). The ^{35}S-radioactivity of a 0.5 ml aqueous sample added to 8 ml ethozyethanol and 8.4 ml of toluene containing 12 g of PPO (2, 5 -diphenyl oxazole) per liter of solution was evaluated in a liquid scintillation counter.

RESULTS:

Comparison of the data shown in Table 6 shows that when rats were fed diets containing the same level of total sulfur as sulfate (diets 1, 3, and 4), adding 0.1% of sulfate to the diet decreased the excretion of the ^{35}S of ^{35}S-cysteine as ^{35}S-taurine by 58%. Adding 0.42% sulfate to the diet (diet 4) decreased the ^{35}S-taurine excretion by 82%. The latter figure is most likely a reflection of both inorganic sulfate addition and cysteine restriction since diet 4 had no added cysteine. However, when cysteine supplementation was constant (diets 2, 3, and 5), addition of 0.1% and 0.42% of sulfate to diets containing equal levels of cystine decreased ^{35}S-taurine excretion 52 and 34%, respectively. The difference in the percentage decrease between diets 4 and 5 is probably a reflection of the increased total sulfur as sulfate in the diet. Diet 5 contained 0.99% of total sulfur as sulfate compared to 0.67% in diet 4.

These data have shown an increase in taurine excretion by those rats forced to satisfy their sulfate requirements by oxidizing cysteine sulfur. Excretion of excess taurine has been shown by Anthony et al. (14) and indirectly by Huxtable and Bressler (15). Since the ratio of glycocholate to taurocholate (G:T ratio) in the intestine may be altered by the available taurine (16), and since—as shown by the previous experiment—the urinary excretion of taurine may be altered by the level of inorganic sulfate in the diet, it seemed mandatory to determine if the G:T ratio could be altered by the level of inorganic sulfate in the diet.

Effect of Dietary Inorganic Sulfate on the Glycocholate: Taurocholate Ratio

A relationship between the conjugation of cholic acid with taurine and the regulation of serum cholesterol was implied by the investigations of Mann et al. (17, 18). They observed that a number of compounds were effective in reducing hypercholesterolemia in monkeys fed a sulfur-deficient diet. One commonality between these compounds was that they were organic sulfur-containing compounds which had a precursor relationship to taurine.

The relative conjugation of cholic acid with glycine and taurine (G:T ratio) has been related to the regulation of serum cholesterol levels (19). The dog and the rat are two animals with a normally low G:T ratio, while the rabbit has a relatively high G:T ratio. The dog and the rat can control their serum cholesterol levels when fed added dietary cholesterol, but the serum cholesterol levels of the rabbit becomes uncontrollable upon the ingestion of dietary cholesterol. In the human the G:T ratio changes throughout the life cycle (20) and can be altered by diet (16). Oral administration of a taurine load causes increased taurine conjugation and a decrease in the G:T ratio of humans. Therefore the following experiment was designed to determine the effect of alterations in dietary sulfate on the G:T ratio and serum cholesterol levels.

The Effect of Dietary Sulfate on the Glycocholic: Taurocholic Acid Ratios and Serum Cholesterol

PROCEDURE:

Nine groups, including 45 adult rats of the Sprague Dawley strain, were fed the following diets: 0.0002% of sulfate shown in Table 1 with 0.53% of cysteine added (diet 1), and 0.40% of sulfate added (diet 2); the 0.10% sulfate diet (diet 3), and the 0.42% sulfate

diet with 0% of added cysteine (diet 4), and 0.40% of added cysteine (diet 5). Diets, 1, 3, and 4 contained equal amounts of total sulfur expressed as sulfate (0.67%) but different levels of organic sulfur. However, diets 2, 3, and 5 contained equal levels of organic sulfur but different levels of total sulfur expressed as sulfate. Cholesterol was added to all diets at a level of 0.1% by dissolving the cholesterol in the melted fat.

At the conclusion of the 17-day dietary treatment period, the rats to be used for analyzing the G:T ratio were administered 50 mg of ^{14}C-carboxy sodium cholate with a specific activity of 8.4×10^5 counts/min/mg. This amount of ^{14}C-carboxy sodium cholate is sufficient to get representative samples of ^{14}C-glycocholate and ^{14}C-taurocholate in the intestine.

The rats were killed 24 hours after feeding the ^{14}C-carboxy cholic acid. The rats' small intestines were removed, frozen, and stored at -20° until the bile acids could be extracted. Bile acids were extracted from the thawed intestine and its contents by the method of Norman and Sjovall (21), separated on thin-layer chromatograms by the method of Anthony and Beher (22) using the solvent system of Hoffman (23), and visualized by the method of Randerath (24). The appropriate spots were scraped from the plates into liquid scintillation counting vials and their radioactivity determined. Free and total cholesterol were determined by the method of Brown et al. (25). The latter value must be determined following alkaline hydrolysis of the cholesterol esters (26).

RESULTS:

The data in Table 7 show the effect of feeding rats diets containing different levels of total sulfur as sulfate and different inorganic to neutral sulfur ratios on the G:T ratio in the small intestine. Rats fed the diet containing 0.1% of sulfate (diet 3) had the smallest G:T ratio. Rats fed the diet containing 0.42% of inorganic sulfate but no added cysteine (diet 4) had a very high G:T ratio—7,730% higher than that of rats fed diet 3; adding 00.4% cysteine to this diet (diet 5) lowered the G:T ratio but not to the low level of diet 3. Removing 0.13% of cysteine from diet 1 raised the G:T ratio 42%.

The data shown in Table 8 show the effect of dietary sulfate, levels of sulfur, and ratios of neutral to dietary inorganic sulfate on the level of serum cholesterol. These data show a significant elevation in both free and total serum cholesterol in rats fed diet 4, the diet in which the G:T ratio was very high (Table 7).

The lowest G:T ratio obtained in this investigation was observed in rats fed diet 3, a diet containing 0.1% of sulfate and 0.40% of cysteine supplementation. Removal of the inorganic sulfate raised the G:T ratio 160% (diet 2). Adding 0.13% of additional cysteine

Table 7. A comparison of G:T ratios of rats fed diets with different levels of total organic and inorganic sulfur

Diet no.	Dietary sulfate	Cysteine added	Glycocholic: increase[1]	Taurocholic acid ratio decimal equivalent
	%	g/100 g	%	
1	0.0002	.53	83	0.086[2]
2	0.0002	.40	160	0.122
3	0.1	.40	--	0.047
4	0.42	.00	7,730	3.68
5	0.42	0.40	91	0.090

[1]Calculated from diet 3.

[2]Values represent averages of duplicate determinations with five rats.

Table 8. Serum cholesterol values of rats fed varying levels of inorganic sulfur, organic sulfur, and total sulfur as sulfate

Diet no.	Dietary sulfate	Cysteine added	Serum cholesterol values		
			Total	Free	Esterified
		g/100 g		Mg/100 Ml Serum[1]	
1	0.0002%	53	95	53	41
2	0.0002%	40	105	40	65
3	0.1%	40	94	49	45
4	0.42%	0	131	63	68
5	0.42%	40	102	52	50

[1]Values are the averages of duplicate determinations on the serum from four rats.

lowered the G:T ratio 30% (diet 1) but not to the low level of diet 3. The 0.13% of additional cysteine could be oxidized to sulfate to supply endogenous sulfate equivalent to the 0.1% of sulfate in diet 3. It is apparent that these data support those presented earlier (Tables 2 and 6), confirming that endogenous cysteine is not a physiologically economic source of endogenous sulfate. Although cystine addition did not replace the need for exogenous inorganic sulfate, cystine restriction increased the G:T ratio and the serum cholesterol levels. Therefore these data show that the level of sulfate and of sulfate and taurine precursors in the diet may play an important and equal role in the dietary management of hypercholesterolemia.

The data which are shown in Table 9 are a repeat of the data shown in Tables 6 and 7; however, when they are arranged in this manner, they provide an easy comparison to check the reliability of our theory that increased urinary taurine should be reflected in a decreased G:T ratio. The diet which resulted in the lowest urinary taurine excretion (diet 4) also gave the very high G:T ratio; therefore, for large changes in urinary taurine, our theory that urinary taurine levels would reflect the availability of taurine for cholate conjugation appears to be correct.

Table 9. Comparison of urinary taurine excretion and degree of taurine conjugation of rats fed varying levels of inorganic sulfur, organic sulfur, and total sulfur as sulfate

Diet no.	Dietary SO_4	Cysteine added	Urinary taurine	Diet[1] no.	Decreasing relative amounts of taurocholic acids G:T ratio
	%	g/100 g	CPM x 10^{-3}		
1	0.0002	0.53	3.3	3	0.047
2	0.0002	0.40	2.9	1	0.086
3	0.10	0.40	1.4	5	0.090
4	0.42	0.00	0.6	2	0.122
5	0.42	0.42	1.9	4	3.68

[1]These diets have the same inorganic sulfate and cysteine composition as the corresponding numbers for taurine excretion.

However, it is obvious that factors other than taurine availability must be affecting taurine conjugation. For example, rats fed diet 1 excreted the most taurine but they exhibited an 83% increase in the G:T ratio over rats fed diet 3. Yet rats fed diet 3 had next to the lowest urinary taurine excretion. These two experiments indicate that the level of inorganic sulfate, total sulfate, and neutral sulfur in the diet is affecting not only the level and availability of tissue taurine but other requirements for conjugation as well.

Bremmer (27) has shown that the conjugation of cholic acid with either glycine or taurine requires the presence of COA, ATP, and some divalent cation, Mg^{++} or Mn^{++}. He suggests that conjugation proceeds according to the following reaction scheme:

I. Cholic acid + COA-SH —(Bile acid activating enzyme)→ Cholyl-S-COA + AMP & Pyrophosphate

II. Cholyl-S-COA —(Taurine bile acyl transferase I)→ Tauro-cholic acid + COA-SH

Cholyl-S-COA —(Glycine bile acyl transferase II)→ Glycocholic acid + COA-SH

Therefore, since rats which were fed the diet without added cystine (diet 4) excreted the lowest levels of urinary taurine and had the highest G:T ratio, it is attractive to suggest that dietary sulfate, total sulfate, and neutral sulfur are influencing the tissue concentration of coenzyme A and therefore influencing the conjugation of cholic acid with taurine.

Effect of Dietary Sulfate on the Conjugation of Glycine and Taurine with Cholic Acid

In view of these considerations, an investigation was designed to investigate the effect of different levels of inorganic sulfate, total sulfate, and neutral sulfur on the relative conjugation of glycine and taurine with cholic acid in rat liver microsomal preparations.

PROCEDURE:

Forty adult male Wistar rats from the colony maintained by the Nutrition Department, The University of Tennessee, Knoxville, were divided into two groups of five and fed the 0.0002% sulfate diet containing 0.40% added cysteine (diet 1); the 0.1% sulfate diet containing 0.40% added cysteine (diet 2); the 0.42% sulfate diet containing 0.40% added cysteine (diet 3); and the 0.42% sulfate diet containing no added cysteine (diet 4) as described in Table 1 for a period of 17 days. At the end of the dietary treatment period, the rats were stunned by a blow to the head, decapitated, and their livers removed for isolation of the microsomal fraction.

The microsomal fraction was isolated from a 10% w/v tissue homogenate in a 0.25 M sucrose, 0.01 M KCl, 0.03 M $MgCl_2$ solution which had been centrifuged for 1 minute at 8200 x g. The microsomal fraction obtained from one cellulose nitrate tube was suspended in 4 ml 0.067 M phosphate pH 7.4, and a modification of Bremmer's (27) method was used to determine **in vitro** bile acid conjugates. One ml of this suspension was added to an Erlenmeyer flask containing 4μ moles taurine, 4μ moles 24- ^{14}C-cholic acid, 4μ moles taurine, 4μ moles glycine, 0.30μ moles coenzyme A, 10μ moles ATP, 0.4μ Moles NaF, and 2μ moles $MgSO_4$ in 0.5 ml 0.067 phosphate buffer pH 7.4. After incubating at 37° for 90 minutes in a shaking H_2O bath, the reaction was stopped by immersing the Erlenmeyer flasks in a boiling H_2O bath for 1 minute.

After cooling the flasks' contents, the bile acid conjugates were extracted by adding 2 ml 1-butanol and shaking for 15 minutes. The samples were then centrifuged at 715 - x g for 10 minutes and the upper layers were collected and evaporated to dryness in a 55° convection oven. The bile acids were reconstituted with 0.2 ml of 1-butanol and were separated, identified, and evaluated for radioactivity as described previously (22, 23, 24). Free coenzyme A was determined in a 10% liver homogenate in 0.1 M Tris buffer pH 8.0 freed of mitochondria and nuclei by centrifugation at 28,700 x g for 10 minutes, using the catalysts method described by Novelli (28). Nitrogen content of the microsomal fraction of the liver homogenate was determined by the micro Kjeldahl procedure (10).

The data which are presented in Table 10 show that if the reaction is run with added coenzyme A, a dietary effect on the G:T ratio is observed only with diet 1, a diet low in inorganic sulfur. The effect observed is an approximate 100% increase in the average G:T ratio of those rats fed diets without any inorganic sulfate.

Table 10. Relationship between dietary sulfate and the glycocholic: taurocholic acid ratio in rat liver microsomal preparations with and without coenzyme A fortification

Diet no.	Dietary So_4	Cysteine Added	G:T ratio	
			With added COA-SH	Without added COA-SH
	%	%		
1	0.0002	0.40	0.21[1]	0.56
2	0.1	0.40	0.11	0.32
3	0.42	0.40	0.12	0.39
4	0.42	0.00	0.11	0.61

[1]Values are averages of duplicate determinations of microsomal fraction from five rats.

Since these data did not duplicate either the data for the amount of taurine in the urine or the G:T ratios obtained from **in vivo** experiments show in Table 9, the assay was run without added coenzyme A. These data (Table 10) do reproduce the data obtained from the **in vivo** experiments: those diets which produced the highest G:T ratio in the **in vivo** experiment—the 0.0002% of sulfate and 0.4% of cystine (diet 2, Table 8 and diet 1, Table 10) and the 0.42% of sulfate and 0.0% of cystine (diet 4, Table 8 and diet 3, Table 10)—also produced the highest G:T ratio with the microsomal enzyme preparation. Therefore, these data seem to indicate that in the intact animal, the phenomenon which is influencing the G:T ratio most is the availability of tissue coenzyme A.

The data which are shown in Table 11 may be used to strengthen the suggestion that coenzyme A availability may be the controlling factor in determining the G:T ratio. Although these data show a decrease in both glycocholate and taurocholate formation in the absence of added coenzyme A, the percentage decrease was greater (85%) for the formation of cholic acid than for the formation of glycocholic acid (55%). In addition, the total amount of glycocholate formed was approximately 0.1 that of taurocholate formation. The net result of these observations is that running the enzyme assay without added COA increases the G:T ratio from an average of 0.15 to an average of 0.28 or about double the G:T ratio obtained with added coenzyme A.

Table 11. Effect of different levels of dietary sulfate on the formation of ^{14}C-glycocholate on ^{14}C-taurocholate in rat liver microsomal preparations with and without coenzyme A fortification

Diet[1] no.	^{14}C-glycocholate formed		^{14}C-taurocholate formed	
	With CoA	Without CoA	With CoA	Without CoA
	Moles per milligram of nitrogen			
1	5.7[2]	2.3	43	7.0
2	5.8	2.4	51	8.3
3	6.3	2.2	63	6.8
4	5.3	2.5	50	15

[1]Diet no. corresponds to the level of dietary sulfate 1 = 0.0002%, 2 = 0.10%, 3 = 0.42%, 4 = 0.42% + 0.4% cystine.

[2]Values represent averages of duplicate determinations from four rats.

Actual tissue coenzyme A levels presented in Table 12 further strengthen this concept since those rats fed diets 1 and 2 had the lowest tissue COA levels as well as the highest G:T ratios.

These data have indicated that when both taurine and glycine are available in a reaction mixture, taurine is the preferred substrate

Table 12. Effect of different levels of dietary sulfate on the level of free coenzyme A in a mitochondrial free rat liver homogenate

Diet no.	Dietary SO_4	Cystine added	Free coenzyme A in liver
	%	%	μg/g
1	0.0002	0.4	31
2	0.1	0.4	32
3	0.42	0.0	31
4	0.42	0.4	36

for conjugation (Table 11). Literature reports (27, 29) indicate that there are two enzymes, one for glycine and another for taurine conjugation. If so, then taurine should not be the preferred substrate when both glycine and taurine are present in the incubation mixture in equal molar concentration.

In an attempt to resolve this apparent discrepancy, Michaelis constants for bile acyl transferase were determined using the Lineweaver and Burk (30) plot of the reciprocal of the initial velocity vs the reciprocal of the substrate concentration. These data (Table 13) show that the Michaelis constant for glycine is about 15 times the Km for taurine, i.e., 15 times more glycine than taurine would be required to run the enzyme at half maximum velocity. When taurine was added to the incubation mixture, the Km for glycine was increased an additional 10 fold but there was no change in the 1/v intercept. These data indicate that taurine is a competitive inhibitor of glycine conjugation.

Table 13. Michaels constants [Km] of the enzyme bile acyl transferase for glycine and taurine and glycine + taurine

Substrate	Km molar
Taurine	2.2×10^{-4}
Glycine	3.3×10^{-3}
Glycine + 6.6×10^{-7} M taurine	2.5×10^{-2}

To further demonstrate the non-specificity of the enzyme bile acyl transferase, β-alanine—a structural analog of taurine—was tested for conjugation by this enzyme system. These data (Table 14) show that bile acyl transferase catalyzed the conjugation of cholic acid with β-alanine but at a rate about half that of glycine or taurine. These data further show that adding taurine to the reaction mixture competitively inhibits the conjugation of both glycine and β-alanine, but that neither of these substrates inhibited the conjugation of taurine.

Table 14. Effect of different substrates on ^{14}C-cholic acid conjugation

Substrate	^{14}C-taurine conjugate	^{14}C-glycine conjugate	^{14}C-β-alanine conjugate
	μmoles/mg N		
Taurine	10.64		
Glycine	---	15.04	---
β-alanine	---	---	6.75
Taurine + glycine	15.02	1.34	---
Taurine + β-alanine	15.85	---	0.66
Glycine + β-alanine	---	15.04	4.72

These data (Tables 10-14) may be used to explain the apparent discrepancy between the urinary excretion of taurine and the G:T ratio in the intestines of rats fed diets containing different sulfate levels and neutral to inorganic sulfate ratios (Table 9).

Literature reports (31, 32) have indicated that the sulfur nutritional status of an animal may affect the tissue level of coenzyme A. When coenzyme A was omitted from the reaction mixture in these experiments (Table 10), the G:T ratio was increased in those microsomal preparations from rats fed diets deficient in either cysteine or sulfate. These same diets were also shown to limit liver coenzyme A levels (Table 12).

The data shown in Table 11 indicated that the synthesis of taurocholate is more coenzyme A-dependent than is the synthesis of glycocholate. The combined data allow the following explanation for the apparent discrepancy between urinary taurine excretion and intestinal G:T ratios: The diet which had high urinary taurine and a high G:T ratio (diet 2, Table 9) was also shown to limit liver coenzyme A levels (Table 11). Therefore, even though there were high levels of taurine in these tissues, its relative conjugation was inhibited by the limiting levels of coenzyme A.

In rats fed the other diet with a high G:T ratio (diet 4, Table 9), both taurine and coenzyme A were limiting. This latter observation may explain the very high (3.68) G:T ratio obtained in rats fed diet 4. Therefore, these data combined with those shown in Table 8 show that any dietary treatment which alters the level of taurine and/or coenzyme A in the tissue will also change the G:T ratio and may be important in the dietary management of serum cholesterol levels.

These observations have indicated that inorganic sulfate must be included in the diet of a monogastric animal if certain metabolic alterations are to be avoided; however, they give no information with respect to the optimal level of dietary inorganic sulfate. Therefore, an investigation was conducted to determine if there was an optimal level of inorganic sulfate for the diet of one monogastric animal, the rat.

Determination of the Optimal Level of Dietary Inorganic Sulfate for the Rat

PROCEDURE:

Ninety-two adult (250-300 g) male rats of the Long-Evans strain were fed variations of the diets shown in Table 1, containing 0.0002, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, and 0.42% sulfate with methionine added to keep the total sulfur as sulfate at 0.67% for a period of 7 days. At the end of the dietary period, the rats were given approximately 12 mg of 1-^{14}C-methionine with an average specific activity of 7.7 x 10^6 CPM/mg. In a comparison study, U-^{14}C-cysteine was used with a specific activity of 7.8 x 10^6 CPM/mg. The amino acids were dissolved in 0.01 N HCl and administered as 0.5 ml solutions by stomach tube. Expired CO_2 was collected continuously for 3 hours following administration of the ^{14}C-amino acid. Two rats were placed in separate jars fitted with two-hole stoppers so that air could be drawn, by a water aspirator, into the jars through 0.1 N NaOH traps and out through two banks of three collecting tubes, each containing 30 ml of 20% NaOH. At the end of the 3-hour collection period, the NaOH-Na_2 $^{14}CO_3$ solution from each tube was pooled and duplicate ml-aliquots were taken for ^{14}C-determination. The aliquots were placed in an all glass vacuum still and acidified by the slow addition of concentrated HCl through an embolution tube. Ten ml of a solution of 12 g 2, 5 diphenyloazole (PPO) in a liter of toluene plus 2 ml of Soluene 100 in a counting vial were used to absorb the liberated $^{14}CO_2$. Radioactivity was evaluated as described previously with a liquid scintillation counter.

RESULTS:

The exact fate of the carbon skeleton of methionine remains unknown; therefore a comparison of the $^{14}CO_2$ expiration from 1-^{14}C-methionine U-^{14}C-cysteine was made. These data—which are shown in Table 15—show that 1-^{14}C-methionine is apparently metabolized three times as rapidly as U-^{14}C-hysteine, expressed either as Cpm/g rat or total μ moles $^{14}CO_2$ expired. According to the previously discussed pathways of methionine metabolism (Figure 2), before methionine could lose the carboxyl of its carbon skeleton, homocysteine would have to give its sulfur moiety to serine to form cystathionine then cysteine. Since the carboxyl carbon of methionine was expired three times as rapidly as the carbon atoms of the cystine skeleton, 1-^{14}C-methionine was selected as the amino acid to be used in assessing the optimal level of dietary inorganic sulfate.

Table 15. Comparison of the $^{14}CO_2$ expired in a 3-hour period from 1-^{14}C-methionine and U-^{14}C-cysteine

Amino acid	$^{14}CO_2$ expiration (% dose x 10^2/g rat)	Total U moles[1] $^{14}CO_2$ expired
1 – ^{14}C-methionine	3.6 (4)[2]	10.7
U – ^{14}C-cysteine	1.3 (4)	3.5

[1]Calculated from the specific activity of the ^{14}C-amino acid.

[2]Numbers in parentheses indicate the number of animals used data are means.

Three different levels of dietary inorganic sulfate (0.0002, 0.1, and 0.42%)—which were used routinely in our previous investigations—were tested for their effect on the expiration of $^{14}CO_2$ from 1-^{14}C-methionine. These data (Table 16) show a 23% increase in $^{14}CO_2$ expiration of those rats fed the diet containing 0.0002% inorganic sulfate as compared with that of rats fed the diet containing 0.1% sulfate. Increasing the inorganic sulfate to 0.42% did not result in any further decrease in $^{14}CO_2$ expiration.

Table 16. The effect of dietary inorganic sulfate on the expiration of $^{14}CO_2$ from 1-^{14}C-methionine

Dietary inorganic sulfate	$^{14}CO_2$ expiration
%	(% dose x 10^2/g rat)
Expt. 1:	
0.0002	7.4 (7)[1]
0.1	6.0 (7)
0.42	6.8 (8)
Expt. 2:	
0.05	6.0 (5)
0.10	6.2 (5)

[1]Numbers in parentheses indicate the number of rats used data are means ± SEM.

The rationale for these experiments was the assumption that by forcing a rat to satisfy its sulfate requirements by endogenous synthesis, a methionine deficit would be created (33). Therefore, as inorganic sulfate spares methionine, methionine would be available for other body functions, the catabolism of methionine would be decreased, and less $^{14}CO_2$ would be expired. Based on this assumption, these data (Table 16) show that of the three dietary sulfate levels listed, 0.0002% was inadequate but that 0.1% appeared to be adequate since further increases (0.42%) did not conserve additional methionine.

The $^{14}CO_2$ expiration of rats fed diets containing 0.05 and 0.1% inorganic sulfate were compared to determine if the optimum level of sulfate was between 0.05% and 0.0002% or 0.05% and 0.1%. These data (Table 16, Expt 2) show no significant difference in $^{14}CO_2$ expiration. Therefore, it appears that the optimal level of dietary inorganic sulfate is somewhere between 0.0002% and 0.05%.

The $^{14}CO_2$ expiration of rats fed diets with 0.01% increments of inorganic sulfate added to the diet was then determined (Table 17, Expt 1). These data show a significant reduction ($P \leq 0.05$) in $^{14}CO_2$ expiration when rats fed diets containing 0.02% of inorganic sulfate are compared with those fed 0.01% inorganic sulfate. With the exception of a very low value for rats fed the diet containing 0.04% inorganic sulfate, a significant increase ($P < 0.01$) in $^{14}CO_2$ expiration by rats fed diets containing amounts of sulfate larger than 0.02% of sulfate was also observed.

Table 17. The effect of small increases in dietary inorganic sulfate on the expiration of $^{14}CO_2$ from 1-^{14}C-methionine

Dietary inorganic sulfate	$^{14}CO_2$ expiration (% dose x 10^2/g rat)	
	Expt. 1	Expt. 2
0.01	5.5 (5)[1]	5.7[2] (5)
0.02	3.8 (5)	4.0 (5)
0.03	7.1 (5)	7.1 (5)
0.04	4.4 (5)	6.5 (5)
0.05	6.0 (7)	5.9 (5)

[1]Numbers in parentheses indicate the number of rats used.

[2]Data are means ± SEM.

Since the $^{14}CO_2$ expiration of rats fed the diet containing 0.04% inorganic sulfate appeared to be inconsistent with other data, a second experiment was conducted. These data (Table 17, Expt 2) confirm those of the earlier experiment (Table 17, Expt 1). These data have demonstrated that the optimal level of dietary inorganic sulfate is 0.02% and that if rats are fed diets containing lower or higher amounts than this, changes in the metabolism of the sulfur-containing amino acids will occur. However, the apparent rate of methionine metabolism by rats fed diets containing added sulfate does not reach that of rats fed very low (0.0002%) levels of inorganic sulfate. In addition to establishing an optimal dietary sulfate level for one monogastric animal—the rat—these data support and extend those previously discussed which suggest not only the importance of dietary inorganic sulfate but the importance of the proper amount.

As stated previously, the rationale for these experiments was the assumption that as added dietary inorganic sulfate decreased the demand for endogenous sulfate, methionine would be conserved for other body functions, and therefore less CO_2 would be expired. However, an apparent increase in methionine oxidation with increased levels of dietary inorganic sulfate cannot be explained by this assumption. Instead it appears that sulfate must have some regulatory role in metabolism other than that obviously related to the metabolism of the sulfur-containing amino acids. For example, sulfate has been shown to activate some of the controlling enzymes of carbohydrate metabolism (34, 35). In order to test this concept, a series of experiments were performed to test the effect of dietary inorganic sulfate on metabolic pathways.

The Effect of Dietary Inorganic Sulfate on the Synthesis of Glycogen

PROCEDURE:

Eighteen adult (250-300g) male rats were divided into three groups of six rats each and fed variations of the diets shown in Table 1 containing 0.0002, 0.1, and 0.42% inorganic sulfate for a period of 7 days. At the end of this dietary period, the rats were fasted for 3 hours and given by a stomach tube a pulse dose containing 2mg of U^{14}C-cysteine (specific activity 7.8 x 10^6 CPM/M Mole) dissolved in 0.01N HCl. Three hours later, the rats were killed by decapitation and their livers were quickly removed to 25 ml of hot (boiling water bath) 30% KOH for the isolation of glycogen (36). The ^{14}C-activity of the glycogen was determined as described previously. The purity of the glycogen preparation was determined by use of anthrone reagent (37). And the results were expressed both as counts/min/mg pure glycogen and total counts/min.

RESULTS:

These data which are shown in Table 18 demonstrate that the dietary level of inorganic sulfate does affect the storage of the carbon skeleton of cysteine. These data show that nearly twice as much of the carbon skeleton of cysteine is converted to glycogen by rats fed the optimal level of sulfate (0.02%) as by rats fed diets with dietary inorganic sulfate levels lower (0.0002%) or higher (0.42%) than the optimum. These data then suggest that the controlling enzymes of gluconeogenesis must be activated by high and low levels of dietary inorganic sulfate. Therefore, an experiment was designed to measure the effect of dietary inorganic sulfate on the activity of the controlling enzymes of gluconeogenesis.

Table 18. Recovery of ^{14}C from U-^{14}C-cysteine in liver glycogen of animals fed different levels of inorganic sulfate

	^{14}C-activity in glycogen	
Dietary $SO_4^=$	Counts/min/mg	Total counts/min x 10^{-3}
%		
0.0002	61 (6)[1]	11
0.02	118 (6)	30
0.42	65 (6)	9

[1]Numbers in parentheses indicate the number of animals used.

Effect of Dietary Inorganic Sulfate on the Controlling Enzymes of Gluconeogenesis

PROCEDURE:

Thirty-five adult albino rats of the Long-Evans strain were divided into five groups and fed modifications of the diets shown in Table 1 and in addition, one diet in which the total sulfur as sulfate was not kept constant. This diet was the 0.42% of sulfate diet supplemented with cysteine equal to the cystine supplement of the 0.1% sulfate diet. The rats were fed these diets for a period of 17 days. They were then sacrificed by decapitation and their livers removed to cold Tris buffer (0.1M pH 7.4) for homogenization and enzyme assay. The activity of liver phosphoenol pyruvate carboxykinase, fructose 1-6 diphosphatase, and glucose-6-phosphatase was measured in 10% liver homogenates according to the method of Sillero et al. (38). Liver pyruvate carboxylase activity was determined according to the method of Deodhan and Misty (39). Glycogen synthetase activity of these homogenates was determined according to the method of Villar-Palasi et al. (40). The results are expressed in terms of activity/mg N/min.

RESULTS:

The data which are presented in Table 19 show that higher levels of dietary inorganic sulfate up to 0.1% activate phosphoenol pyruvate carboxykinase. These data provide an explanation for the data which were shown previously (Table 18). The data in Table 18 show a two-fold increase in the storage of the carbon skeleton of cystine as liver glycogen by rats fed diets containing 0.02% inorganic sulfate. In agreement, the data shown in Table 19 show a two-fold increase in the activity of phosphoenolpyruvate carboxykinase.

Since phosphoenol pyruvate carboxykinase activity and pyruvate carboxylase activity are expressed in the same units in Table 19, this increase in phosphoenol pyruvate carboxykinase activity could explain the increased incorporation of the carbon skeleton of cystine into glycogen shown in Table 18—also, the apparent discrepancy observed earlier of a failure of dietary sulfate above 0.02% to spare methionine even though it appeared to spare cystine. These observations were based on the expiration of $^{14}CO_2$ from U^{14}C-cysteine; therefore, if more of the carbon skeleton of cysteine is stored as glycogen, less could be expired as $^{14}CO_2$, but the amino acid would no longer exist to spare methionine.

Table 19. Effects of dietary inorganic sulfate upon the activity of the controlling enzymes of gluconeogenesis

Enzyme: activity:	Percent dietary inorganic sulfate				
	0.0002	0.02	0.10	0.42	0.42+C[1]
Phosphoenolpyruvate-carboxykinase μmoles x 10^{-2}/mg N[2]	1.8	3.9	6.7	5.6	3.2
Pyruvate carboxylase μmoles x 10^{-2}/mg N[2]	16.3	18.2	17.8	20.4	14.2
Fructose 1-6-disphosphatase units/mg N[2]	3.4	3.4	3.3	3.4	3.3
Glucose - 6 - phosphatase[2]	20	20	21	22	20
Glycogen synthetase[2] μmoles/mg N	5.4	5.0	4.6	5.6	5.0

[1]0.42% of dietary inorganic sulfate plus 0.384 g cysteine/100 g of diet.

[2]All data are average values from duplicate determinations with seven animals.

Since these data have shown an effect of dietary inorganic sulfate upon carbohydrate metabolism, and since data presented earlier have shown an effect of dietary inorganic sulfate upon bile acid metabolism—the latter effect due in part to the effect on the level of tissue coenzyme A—it was decided to investigate the effect of inorganic sulfate upon the activity of propionyl-CoA carboxylase. Propionyl-CoA carboxylase is a unique enzyme which catalyzes the formation of a carbohydrate metabolite and glucose, yet, has as its substrate a short-chain acyl derivative of coenzyme A. Therefore, its investigation should serve to further indicate the role of dietary inorganic sulfate as a metabolic regulator.

Effect of Dietary Inorganic Sulfate on the Activity of Propionyl-CoA Carboxylase

PROCEDURE:

Adult male Long-Evans rats were fed variations of the diets shown in Table 1 containing 0.0002, 0.02, 0.10, and 0.42% inorganic sulfate. In addition, the 0.42% sulfate diet—rather than having no

supplemented sulfur-containing amino acid and constant sulfur as sulfate—was supplemented with 0.5% of methionine. All diets were fed for a period of 17 days. At the end of the dietary period, the rats were sacrificed by a blow to the head, decapitated, and their livers removed to cold 0.1M Tris buffer pH 7.2 for homogenization with a motor-driven Thomas size C-homogenizer having a Teflon pestle. Seventy-five ml of the 10% homogenate were placed in a 100 ml beaker and were sonicated for 90 seconds with a Bronwill Biosonik Sonicator set at maximum power and turned for maximum Transducence to remove nuclear and mitochondrial remnants. Ten ml of the clear supernatant fluid were diluted with an equal volume of 0.1M Tris-HCl buffer pH 7.2 and mixed by inversion. The resulting enzyme preparation was used to determine propionyl-CoA carboxylase activity according to the method of Georgio and Whitaker (41). For certain experiments, the above procedure was altered slightly to yield mitochondrial and cytoplasmic preparations. All data were expressed as Nana-Moles/mg N based on micro-Kjeldahl (10) determination of the nitrogen in the homogenate.

RESULTS:

The data which are presented in Table 20 show that if rats are fed diets containing inorganic sulfate levels either below or above the optimum level of 0.02%, the activity of liver propionyl-CoA carboxylase will be increased. This same phenomenon was observed whether propionyl-CoA, sodium propionate, or sodium propionate plus coenzyme A was used as the substrate. Therefore, although based on the decrease in the rate of propionyl-CoA carboxylase activity observed, it would appear that the substances used other than propionyl-CoA are measuring the activity of the acylating enzyme system.

Table 20. Effect of feeding different levels of dietary inorganic sulfate upon the activity of rat liver propionyl CoA carboxylase activity

Dietary inorganic sulfate	Propionyl-CoA substrate	Sodium propionate substrate	Sodium propionate + CoA substrate
%	n moles/mg N[1]		
0.0002	362 (5)[2]	91 (5)	99 (5)
0.02	230 (5)	8 (5)	14 (5)
0.10	388 (5)	74 (5)	110 (5)
0.42 + meth.[3]	454 (5)	43 (5)	121 (5)

[1]Values are means.

[2]Numbers in parentheses indicate the number of rats used.

[3]0.5% of methionine added.

The pattern of increased enzyme activity with levels of dietary inorganic sulfate both above and below the optimum remained the same. It is conceivable that the increase in propionyl-CoA carboxylast activity observed with low levels of dietary inorganic sulfate is a reflection of an attempt to metabolize the carbon skeleton of methionine. Although there may be other metabolites which result from the removal of methionine from the remethylation pathway (33), oxidation to propionate has been suggested as one fate of the carbon skeleton of methionine (42). Therefore, as previously observed (Table 17), the 20% increase in methionine metabolism observed with the diet low in sulfate (0.0002%) compared to those rats fed the optimal level (0.02%) could require an increase in propionyl-CoA carboxylase activity. However, the magnitude of the increase in propionyl-CoA carboxylase (approximately 58%) is larger than would appear to be required and does allow additional interpretations. A similar explanation could be advanced for the increase in diets with inorganic sulfate levels above 0.02%. However, since the magnitude of the increase in activity is greater for rats fed diets containing levels of dietary inorganic sulfate above the optimum, an alternate explanation was sought.

Murthy and Misty (43, 44) have investigated a cytoplasmic factor or factors which are involved in the synthesis of propionyl-CoA holo-carboxylase and the activation of propionyl-apocarboxlase. The data which are presented in Table 21 represent an attempt to investigate the effects of dietary inorganic sulfate on the cytoplasmic factor necessary for activation of the apoenzyme and/or synthesis of the holo-enzyme of propionyl-CoA carboxylase. These data show that combination of the cytoplasm from livers of rats fed 0.0002% sulfate activates the mitochondial propionyl-CoA carboxylase from the livers

Table 21. Effect of feeding different levels of dietary inorganic sulfate upon the availability of a cytoplasmic factor necessary for propionyl-CoA carboxylase synthesis

Dietary inorganic sulfate fed donor rat		Propionyl - CoA carboxylase activity	
Liver mitochondria	Liver cytoplasma	Without biotin	With biotin
SO_4%	SO_4%	N moles/mg	N moles/mg
0.0002	0.0002	13.8	24.2
0.0002	0.02	11.2	31.1
0.02	0.0002	12.9	24.8
0.02	0.02	9.2	34.0

[1] 0.5 μ moles biotin was added to reaction mixtures.

of rats fed diets containing 0.02% inorganic sulfate. And, in agreement, combination of cytoplasm from the livers of rats fed diets containing 0.02% sulfate inhibits the activity of mitochondial enzyme from rats fed 0.0002% sulfate diets.

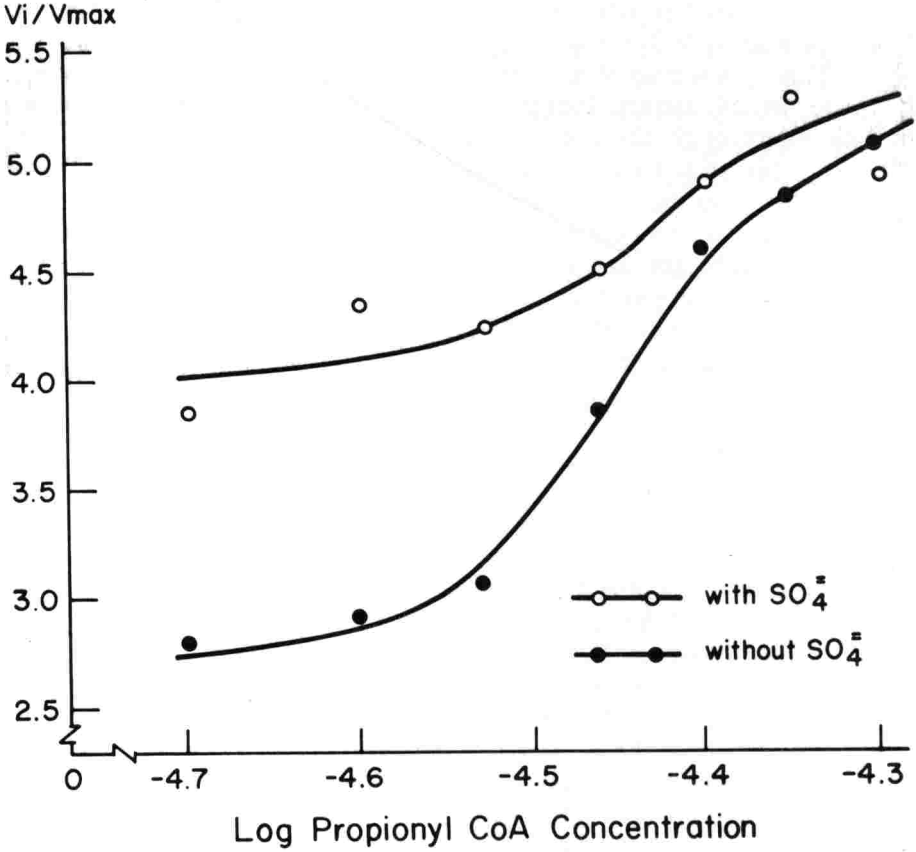
These data suggest that the control of enzyme activity between low levels of dietary inorganic sulfate and the optimum level is mediated through the cytoplasmic factor. The other data presented in this table with biotin added to the reaction mixture allow an expansion of this interpretation. These data—which show the cytoplasmic fraction to be inhibitory to enzyme activity in the presence of exogenous biotin, and which show the highest enzyme activity was obtained with preparations from rats fed optimal levels of dietary inorganic sulfate—suggest that low levels of inorganic sulfate may increase the availability of biotin by a mechanism as yet unknown.

The data contribute little to an explanation of the effect of higher levels of dietary inorganic sulfate on the activity of propionyl-CoA carboxylase. Other investigators have shown an increase in the activity of glucose dehydrogenase (45) and a protection of avidin inhibition of pyruvate carboxylase by adding inorganic sulfate (46). The data which are presented in Table 22 show the effect of adding inorganic sulfate to the reaction mixtures for the assay of propionyl-CoA carboxylase activity. These data shown that adding sulfate to the incubation mixture activates propionyl-CoA carboxylase both when biotin is added to the reaction mixture and when it is omitted. When biotin is added to the mixture, smaller amounts of inorganic sulfate—1.81 μ moles compared to 5.62 μ moles—achieve significant activation.

Table 22. Effect of in vitro inorganic sulfate upon the activity of rat liver propionyl-CoA carboxylase

	Propionyl-CoA carboxylase activity	
Conc of added sulfate	Without biotin	With biotin
μ moles/tube	N moles/mg N	N moles/mg N
0.00	297	311
1.87	295	326
3.75	301	327
5.62	305	340
7.50	322	341
15.00	329	
22.50	336	
30.00	337	

Figure 4. Substrate-saturation curves of propionyl-CoA carboxylase in the presence and absence of sulfate.



Kaziro et al. (47, 48) have shown that propionyl-CoA carboxylast is composed of four identical subunits that undergo conformational changes by the binding of substrates and/or effectors (49). Therefore, it seemed reasonable to determine if higher levels of sulfate were acting as an allosteric effector for propionyl-CoA carboxylase. These data, Figure 4, which show a sigmoid response for the substrate-saturation curve of propionyl-CoA carboxylase in the absence of inorganic sulfate and which show the change in sigmoid response curve in the presence of added inorganic sulfate, fulfill the requirements for an allosteric effector (50). Therefore, it appears that the effects of inorganic sulfate on propionyl-CoA carboxylase may be mediated both through effects on biotin metabolism and allosteric-induced conformational changes. Since the conformational

changes could be related to the ease of biotin binding to the apoenzyme, it is conceivable that the allosteric effect is an expression of biotin sulfate interaction also.

Since these data have shown that the activity of controlling enzymes of carbohydrate synthesis may be affected by the level of dietary inorganic sulfate and/or the ratio of neutral sulfur to sulfate in the diet, and an effect on an enzyme which might bridge carbohydrate and lipid metabolism, it was decided to measure the effect of dietary inorganic sulfate on the controlling enzymes of lipid metabolism.

Effect of Dietary Inorganic Sulfate on the Activity of Acetyl-CoA Carboxylase, Citrate Cleavage Enzyme, and Malic Enzyme

PROCEDURE:

Adult male rats of the Long-Evans strain were fed variations of diets described in Table 1 containing 0.0002, 0.02, and 0.42% inorganic sulfate. In some experiments the added sulfur-containing amino acid was not added at a level so that the total sulfur as sulfate remained constant. The rats were fed these diets for a period of 17 days; then the rats were sacrificed and their livers removed to a cold buffer solution for subsequent removal of nuclei and mitochondria. Enzyme activity of the cytoplasmic preparation was determined. Malic enzyme activity was determined by the method of Ochoa (51). Citrate cleavage enzyme activity was evaluated according to the method of Cottam and Siere (52) and acetyl CoA carboxylase activity was determined as described by Inoue and Sowenstein (53). Nitrogen was determined as described previously (10) so that the results might be expressed in terms of units/mg N.

RESULTS:

The results of this experiment which are summarized by the data presented in Tables 23 and 24 show an effect of dietary inorganic sulfate on the activity of all three enzyme systems evaluated. In each case the activity of malic enzyme was increased by diets low in inorganic sulfate while acetyl-CoA carboxylase activity was decreased with diets low in inorganic sulfate and variable levels of methionine but not lower than 0.02% with constant levels of methionine. In each case, citrate cleavage enzyme was highest in rats fed normal levels of inorganic sulfate, an observation just opposite to those for propionyl-CoA carboxylase (Table 21). And in each case, acetyl-CoA carboxylase activity was highest in those rats fed 0.42% inorganic sulfate. The latter data are compatible with those obtained

Table 23. Effect of dietary inorganic sulfate on the activity of malic, citrate cleavage, and acetyl-CoA carboxylase enzyme activity in rat liver cytosol preparations

Dietary inorganic sulfate	Malic enzyme	Citrate cleavage enzyme	Acetyl-CoA carboxylase
%	unit/mg N/min		
0.0002	10	290	0.28
0.02	6	304	0.36
0.42	9	295	0.42

Table 24. Effect of dietary inorganic sulfate and constant levels of methionine[1] on the activity of malic, citrate cleavage, and acetyl-CoA carboxylase enzyme activity in rat liver cytosol preparations

Dietary inorganic sulfate	Malic enzyme	Citrate cleavage enzyme	Acetyl-CoA carboxylase
%	unit/mg N/min		
0.0002	7	215	0.38
0.02	5	225	0.33
0.42	5	178	0.52

[1] 0.4% of DL methionine added to all diets.

for phosphoenolpyruvate carboxykinase (Table 19) which show a decrease in activity for rats fed diets containing 0.42% inorganic sulfate.

Together, then, these data show that as carbohydrate synthesis decreases, fat synthesis should increase based on controlling enzyme activity. However, these data do open to question the source of the acetate and the reducing equivalents for fat synthesis. In summary or nevertheless, the Tables (19, 24) do show a role for inorganic sulfate as a controlling factor in carbohydrate, lipid, and related metabolism.

These data have shown the importance of inorganic sulfate as a metabolic constituent. And they have provided some indication of an interrelationship between dietary inorganic sulfate and the sulfur-containing amino acids. Especially significant was the role of the optimal level of inorganic sulfate in sparing methionine and the controlling action of dietary sulfate upon the storage of the carbon skeleton of cysteine as glycogen. The latter effect was explained by the activation of the controlling enzymes of gluconeogensis by dietary inorganic sulfate.

An observation of some rats intended for use on avitaminosis E experiments enabled us to extend these observations. Rats fed diets containing 0.0002% sulfate (Table 1) from 2 weeks of age suddenly developed a gross and dramatic lesion of the hind leg which appeared to be due to a malformation of the joints. However, if cod liver oil was added to the diets, the malformation did not appear. This observation led us to an investigation of the effect of cod liver oil on sulfur metabolism.

Effect of Cod Liver Oil on Sulfur Metabolism

PROCEDURE:

Weanling rats of the Wistar strain were fed modifications of the diets shown in Table 1 in which the fat was either "stripped lard" or cod liver oil. The animals were raised on these diets until 2 weeks of age. In order to do this, the pups and their mothers were fed these diets 1 week before weaning. Specific activity of costal cartilage sulfomucopolysaccharide was determined by giving the rats a subcutaneous injection of approximately 5 μc of sodium sulfate ^{35}S 24 hours prior to sacrifice. Sulfomucopolysaccharides were prepared from the costal cartilage by the method of Bostrom (54). The sulfate content of the mucopolysaccharides was determined following hydrolysis (55) by a colorimetric procedure (56). The tibia, fibula, and femur were prepared for histological examination by standard techniques (57). In order to provide test material for various diets, cod liver oil was fractionated by the method of Chen et al. (58) or by the method of Malone and Stoffyn (59). Total sulfur as sulfate in the lung tissue of these animals was determined by the method of Roe et al. (60). Lipid phosphorus was determined as described by Allen (61). Oxidation of methionine ^{35}S sulfur to ^{35}sulfate was compared by incubating 0.1 ml of denucleated 10% liver homogenates in a 50 ml Erlenmeyer flask containing 200 μ moles ^{35}S-methionine 10 μ moles ATP, 600 μ moles $MgCl_2$, and enough 0.06%M phosphate buffer to make 4 ml of final solution. The samples were incubated in a shaking H_20 bath at 37° for 30 minutes. The reaction was stopped by adding 5/ml of 10% trichloroacetic acid. The precipitate was separated by centrifugation and the centrifuge cake was washed with 5 ml of distilled H_20. The supernatant fluids were combined and freed of sulfur-containing amino acids by passing through a Dowex 50 - X 8 (200-400 mesh) Column 1 cm in diameter, 4 cm high. The eluate was evaluated for ^{35}S-sulfate activity (6).

RESULTS:

The rats shown in Figure 5 show the type of lesion which developed in those rats fed the diets without cod liver oil The growth curves shown in Figure 6 show an abrupt break in the growth curve for rats fed the diets low in sulfate but without cod liver oil. The data in Table 25 show that if cod liver oil is not added to these diets, there is a decrease in the percent of sulfur in the mucopolysaccharides isolated from the costal cartilage of the rats. These data also show that the specific activity of these preparations varied without the total counts per minute varying.

This observation is additional proof that the percentage of sulfur in the cartilage mucopolysaccharides is decreased in those rats not receiving cod liver oil in their diet. Further evidence of alterations in sulfur metabolism by those rats fed diets low in sulfate is offered by the photographs shown in Figures 7 and 8. The photographs shown in Figure 7 show that the tibial epiphyseal cartilage was thinner in rats fed diets low in sulfate without cod liver oil than were those of rats fed diets containing inorganic sulfate. And the autoradiogram shown in Figure 8 shows that the chondrocytes were slower in losing a test dose of $^{35}SO_4$ than were those from rats fed diets containing cod liver oil.

Figure 5. Photograph of rats fed a diet low in sulfate with cod liver oil (left) and without cod liver oil (right) showing the characteristic leg lesions of rats fed these diets without cod liver oil *

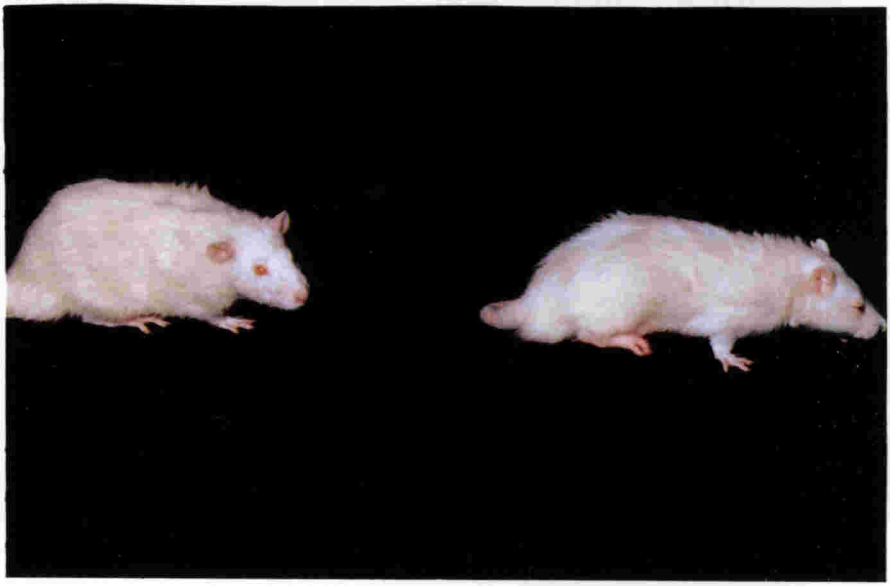


Figure 6. Growth curves for male rats fed a laboratory stock diet and two semi-purified diets, one of which contained cod liver oil and another which did not.

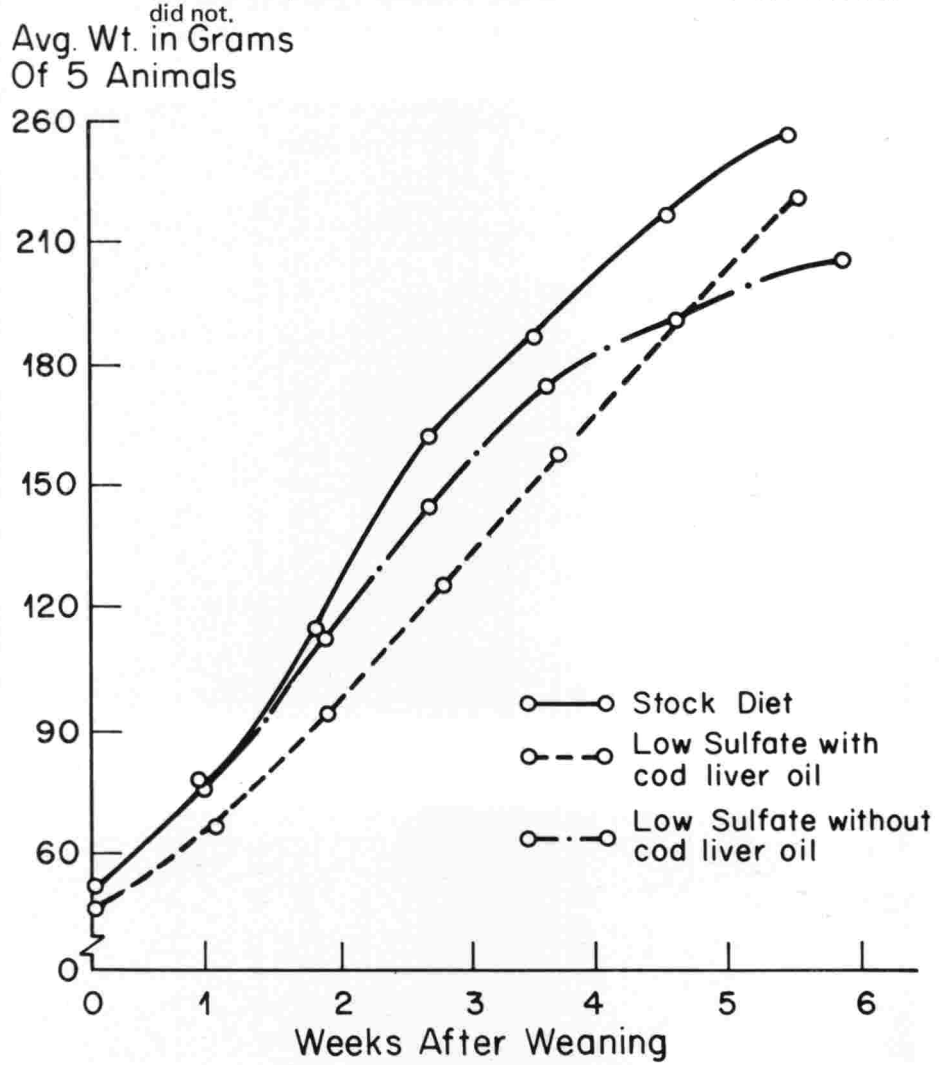


Table 25. Sulfate content of the mucopolysaccharide from pooled samples of costal cartilage

Diet	Sulfate		Mucopolysaccharide			
	Percent		c.p.m./mg x 10^{-3}			
			Sulfate		Total	
	♂	♀	♂	♀	♂	♀
Without cod liver oil	12.3	10.7	11.6	10.6	1.4	1.1
With cod liver oil	15.2	13.6	9.4	9.8	1.4	1.3

Figure 7. Photomicrographs of the Proximal Tibial Epiphyseal Cartilages of male rats fed diets low in sulfate with cod liver oil (top picture) and without cod liver oil (lower picture). 45x magnification.

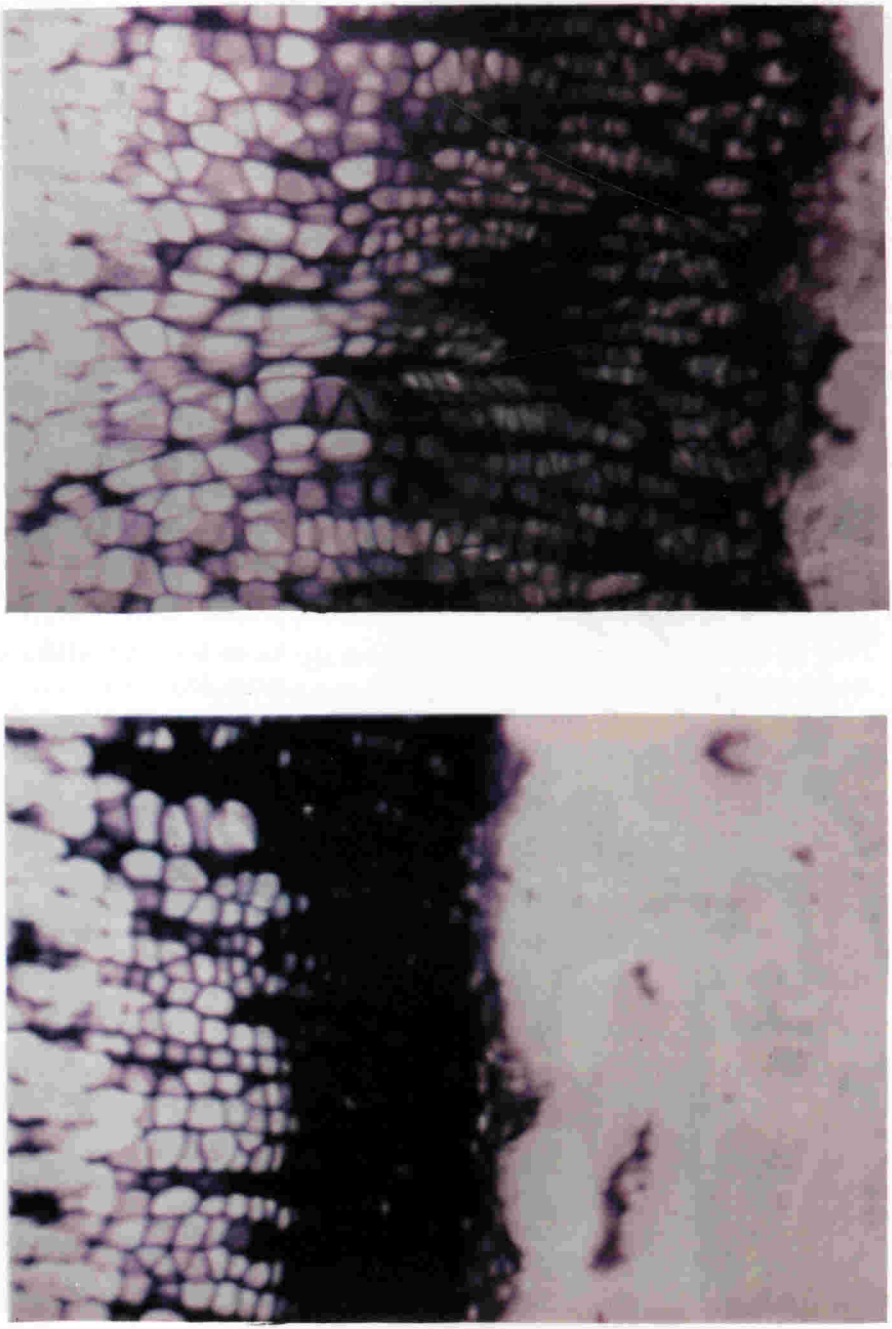
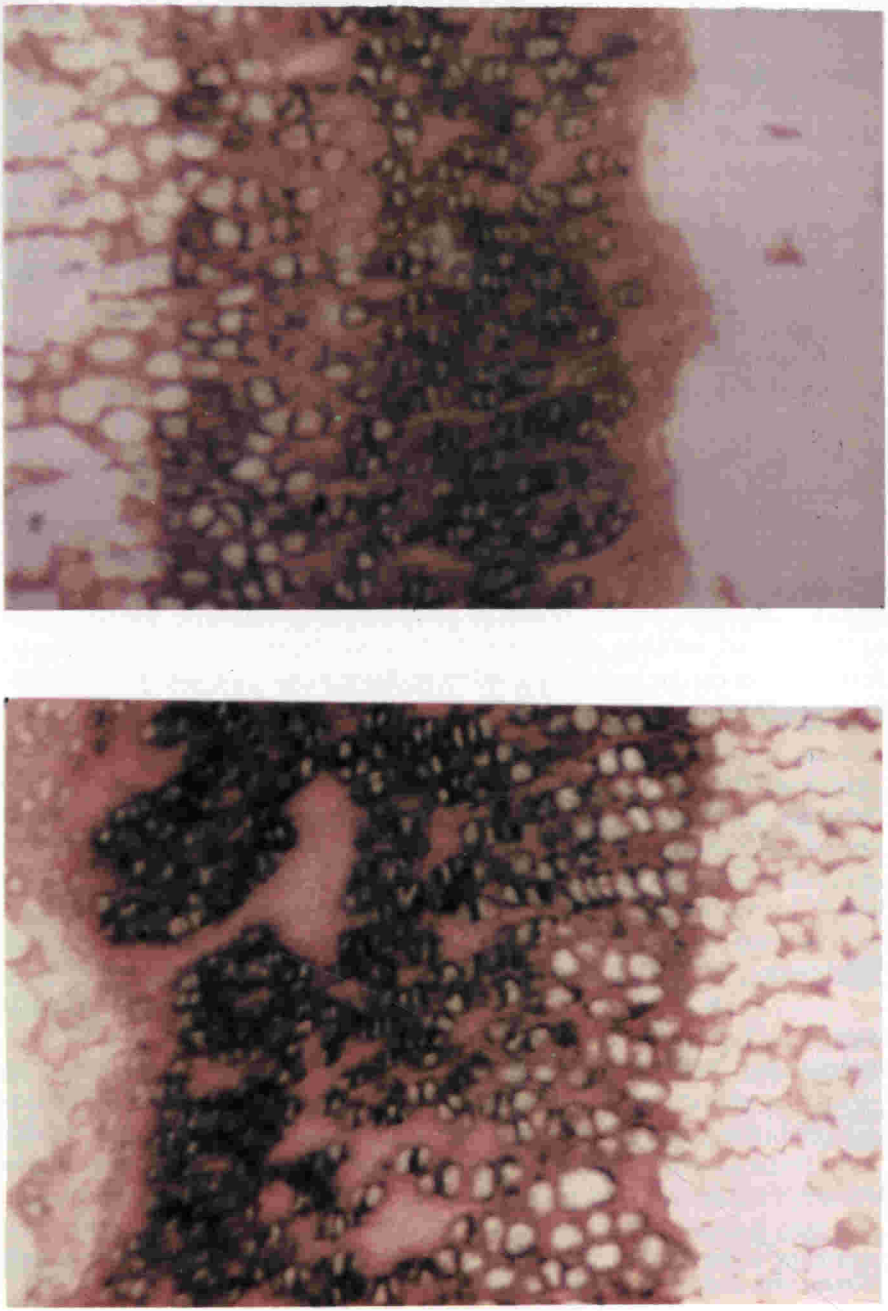


Figure 8. Autoradiophotomicrographs of the Proximal Tibial Epiphyseal Cartilages of male rats fed diets low in sulfate with cod liver oil (top picture) and without cod liver oil (lower picture). Three weeks exposure. Neutral red stain through stripping film. 65x magnification.



These data demonstrated that cod liver oil was beneficial to rats fed diets low in sulfate and supplemented with methionine. It was attractive to speculate that cod liver oil contained a fraction which was necessary for optimal utilization of sulfate. Fractionation of cod liver oil and subsequent incorporation of these fractions into rat diets suggested that the "active" ingredient in cod liver oil was associated with the lipid fraction.

The data which are shown in Table 27 allow a comparison of the effect of cod liver oil and known lipid components. These data show that rats fed diets supplemented with safflower oil most closely mimic those fed diets supplemented with cod liver oil. Lombardi et al. (62) have shown that dietary, long-chain, highly unsaturated fatty acids will influence the pathway of lecithin biosynthesis towards the methylation of phosphatidylethanolamine.

The data in Table 26 show that adding 5% cod liver oil to the diet of a rat as a source of long-chain, highly unsaturated fatty acids increased the rate of metabolism of methionine. There was a 28% increase in ^{14}C-methyl taken up by liver phospholipids, a 34% increase in $^{14}CO_2$ expired per gram of rat, and a 25-30% increase in ^{35}S-sulfate formation from ^{35}S-methionine. Based on these data and those of Lombardi et al., (62) it appears that this increase in methionine metabolism is due to the creation of a methyl demand. By forcing the biosynthesis of lecithin to follow the phosphatidylethanolamine pathway, three moles of S-adenosylmethionine are used for each mole of lecithin synthesized. As a result, more homocysteine is formed than can be remethylated—resulting in an increase in substrate for cystathionine synthetase.

Table 26. Effect of different lipid fractions on feed efficiency, weight gain, uptake of $^{35}SO_4^=$, total sulfur as sulfate

Diet	Feed efficiency[1]	Wt. Gain	Mucopoly-saccharide	Total sulfur as sulfate in mucopoly-saccharide
	%	g	c.p.m./m mole $SO_4^=$	μg $SO_4^=$/mg
Without cod liver oil	25	90	850	7.8
With cod liver oil	28	96	990	14.7
Safflower oil	27	106	1480	12.7
Linolenic acid	27	84	970	12.2

[1]Grams gain divided by grams feed consumed times 100.

Table 27. Effect of dietary cod liver oil on the expiration of $^{14}CO_2$ from 1-^{14}C-methionine, the uptake of ^{14}C by liver lipids from ^{14}C-methyl-methionine, and the oxidation of the sulfur of ^{35}-methionine to ^{35}-sulfate

Diet	$^{14}CO_2$ expired[1]	^{14}C uptake[2]	---------- $^{35}SO_4^=$ formed[3] ----------	
	% dose x 10^{-2} g/rat	c.p.m./min/ mg P	total μmoles	μmoles/ mg N
With cod liver oil	43	18.3	83.2	46.5
Without cod liver oil	32	14.3	64.1	37.2

[1]Female rats given a pulse dose of ^{14}C-methionine specific activity 7.7 x 10^6 c.p.m./m mole.

[2]Female rats given a pulse dose of ^{14}C-methyl methionine specific activity 9.8 x 10^6 c.p.m./m mole.

[3]^{35}S-methionine was incorporated into incubation mixtures specific activity, 3.1 x 10^7 c.p.m./m mole.

The increase in endogenous cysteine which results allows an increase in the oxidation of cysteine sulfur to sulfate (Table 27). These data show that dietary manipulations with long-chain unsaturated fatty acids may increase the conversion of methionine sulfur to cystine sulfur to endogenous sulfate to relieve the sulfur shortage in rats fed diets low (0.0002%) in inorganic sulfate. In addition, these data provide additional evidence to use in questioning the role of cysteine in regulating the metabolism of methionine (33).

As further proof of the metabolic inequivalency of methionine and cysteine, the data which are shown in Table 28 show that the activity of two gluconeogenic rat liver enzymes—phosphoenolpyruvate carboxykinase and pyruvate carboxylase—and an enzyme involved in detoxication reactions, UDP glucuronyl transferase—is quite different depending upon the identity of the sulfur-containing amino acid used to supplement the diets. Supplementation of these diets with equimolar cysteine instead of methionine resulted in increased activity of all of these enzymes. The actual percentage stimulation ranged from 24-130%.

These data (Table 28) show that adding a methyl acceptor guanidoacetic acid to rat diets supplemented with methionine activated pyruvate carboxylase and phosphoenolpyruvate carboxykinase more than adding equimolar cysteine. Since guanidoacetic acid addition creates a methyl demand, it is assumed that additional endogenous cysteine would be produced and that the extra cysteine was responsible for enzyme activation. It may be suggested that the gluconeogenic enzymes are activated as a result of cysteine addition

Table 28. Effect of dietary methionine, methionine + guanidoacetic acid, and cysteine on phosphoenol pyruvate carboxykinase, pyruvate carboxylase, and UDP glucuronyl transferase activity

Dietary supplementation	Phosphoenol-pyruvate carboxykinase	Pyruvate carboxylase	UDP-glucuronyl transferase
	--------μ moles x 10^{-2}/mg N----------		n moles P-nitrophenol/ mg protein
Cystine	9.0	22.5	28.4
Methionine	3.9	18.2	19.3
Methionine + guanidoacetic[1] acid	16.5	40.0	—

[1]0.02% guanidoacetic acid added to a methioine-supplemented diet containing 0.02% of added inorganic sulfate.

to metabolize the carbon skeleton, pyruvate, produced from the metabolism of cysteine. However, this argument would not explain the activation of UDP—glucuronyl transferase by cysteine addition. Therefore, since enzymes from different metabolic pathways have been shown to be activated by cystine supplementation compared to methionine supplementation, these data provide strong evidence that methionine and cystine are not metabolically equivalent as dietary supplements.

The data, which are presented in Table 29, provide additional evidence of the metabolic inequivalency of methionine and cysteine as dietary supplements. These data show a slight but significant increase, 11-15%, in both reduced and total glutathione levels in kidney and liver tissues of rats fed diets containing equivalent (molar basis) levels of cystine supplemented in the place of methionine. These data, together with those presented in Tables 27 and 28, indicate that unless a methyl demand is created in an animal, methionine will not leave the remethylation pathway fast enough to supply the endogenous cysteine required for normal body function. These data and those of other investigators (63, 64) indicate that serious consideration should be given to revising the concept of cysteine as a dispensable amino acid.

The data which have been presented have shown differences in enzyme activities, sulfate utilization, taurine excretion, cholesterol levels, collagen formation, and biotin utilization, and have established an optimal level of dietary inorganic sulfate for one monogastric animal, the rat. However, argument could still be advanced that since

Table 29. A comparison of tissue glutathione levels of rats fed diets supplemented with methionine or cysteine

Dietary supplement[1]	Glutathione levels			
	Kidney		Liver	
	Total	Reduced	Total	Reduced
	$\mu g \times 10^{-3}$/g wet tissue			
Methionine	2.0	1.7	3.6	3.5
Cysteine	2.3	1.9	4.0	4.0

[1]Equimolar supplements to a 15% of casein diet containing 0.02% of dietary inorganic sulfate.

animals fed diets abnormal with respect to sulfur nutrition are difficult to tell from those fed diets normal with respect to sulfur nutrition, then is there practical significance to these differences which we have reported? Another way to approach this problem would be to investigate the reaction of an animal fed diets of different sulfur levels to stress induced by drugs, insecticides, or altered vitamin intake.

Effect of Dietary Inorganic Sulfate on Tissue Levels of Natural Constituents Related to Detoxication

PROCEDURE:

Tissue glutathione levels in adult Long-Evans rats fed diets containing different levels of inorganic sulfate and/or sulfur-containing amino acids were determined by the glyoxylase method (65). Since glutathione levels might influence the level of amino acids in the urine, amino nitrogen was determined in urine aliquots by the method of Fisher et al. (66). The ratio of salicylamide conjugated with glucuronic acid to that conjugated with sulfate was determined by the method of Levey and Matsuzawa (67) in urine aliquots from rats fed diets containing 0.5 g of salicylamide per 100 g of diet for 96 hours. Cellular lipoprotein was prepared from the livers of rats by the method of Smith et al. (68) as modified by Levin and Thomas (69). Total sulfur as sulfate in tissues and tissue fractions was determined by the method of Roe et al. (70). Galactose in sulfolipid samples was determined according to the method of Fisher et al. (71). Arylsulfatase activity was determined in liver samples by the method of Roy (72). Total hexosamine was determined in lung tissue by the method of Boas (73).

RESULTS:

The data presented in Table 30 show that the change in glutathione produced by alterations in sulfur nutrition—i.e., dietary supplementation with methionine instead of cystine—does have an effect on the body. Methionine supplementation which produces less glutathione in the kidney also allows more amino acids to remain in the urine. The extra urinary amino acids presumably represent an impairment of the γ-glutamyl cycle (74). The effect of dietary inorganic sulfate (Table 31) on glutathione concentration confirms results presented previously with respect to the optimal level of dietary inorganic sulfate. These data show that rats fed diets with 0.02% inorganic sulfate supplementation (the optimal level) had the highest level of tissue glutathione in the liver. However, in the kidney, increasing dietary sulfate gave a slight increase in glutathione levels. The latter data presumably reflect a different control mechanism in the kidney than in the liver. These data have shown that a dietary stress may change urinary excretion products.

Table 30. Glutathione concentration of kidney cortex and γ-amino nitrogen levels in the urine of rats fed diets containing 0.02% of inorganic sulfate

Dietary supplementation	Reduced glutathione kidney cortex	γ-amino nitrogen in urine
	$\mu g \times 10^{-3}$/g tissue	μmoles/24 hr.
Methionine	2.1	0.62
Cysteine	2.3	0.47

Table 31. Effect of different levels of dietary inorganic sulfate on the levels of glutathione in rat liver and kidney

Dietary[1] sulfate	Total glutathione	
	Liver	Kidney
%	..... $\mu g \times 10^{-3}$/g wet tissue	
0.0002	1.8	0.95
0.02	2.3	0.96
0.1	2.2	1.08
0.42	2.2	1.09

[1]All diets contained 0.4% of added cysteine.

The data presented in Table 32 show the effect of diet in relation to the stress of detoxication reactions. These data also confirm that 0.02% is an optimal level of dietary inorganic sulfate. Adding

Table 32. The effect of dietary sulfate on the glucuronide-salicylamide: sulfate-salicylamide ratio in the urine of rats

Dietary sulfur	Glucuronide-salicylamide: sulfate-salicylamide ratio
0.0002% $SO_4^=$	0.89 (18)[1]
0.0002% $SO_4^=$ + 0.325% methionine	0.99 (5)
0.0002% $SO_4^=$ + 0.65% methionine	0.37 (15)
0.02% $SO_4^=$	0.27 (6)
0.02% $SO_4^=$ + 0.62% methionine	0.35 (19)
0.1% $SO_4^=$	0.25 (6)
0.1% $SO_4^=$ + 0.5% methionine	0.38 (5)
0.42% $SO_4^=$	0.24 (7)

[1]Numbers in parentheses are the number of rats in the study.

0.02% sulfate without further amino acid supplementation to these 15% casein based diets results in a 30% reduction in the glucuronide-salicylamide: sulfatesalicylamide ratio (GS:SS ratio) over that obtained with 0.0002% sulfate and 0.65% methionine. These data also show that no further reduction in the GS:SS ratio was obtained by increasing the level of inorganic sulfate.

Glucuronidation and sulfation are both important detoxication reactions. However, the limiting factor in the formation of salicylamide sulfate appears to be the availability of sulfate (67). Therefore it appears that diets with altered sulfur nutrition (Table 34) are stressing the body by forcing detoxication reactions to utilize glucuronic acid. Since some systems—bilirubin conjugation, for example—prefer glucuronate (75) conjugation and because of the importance of UDP-glucuronate in the formation of mucopolysaccharides and sulfolipids and the role of UDP-glucose in galactose metabolism and glycogen synthesis, it is conceivable that the stress of drug detoxication coupled with inadequate sulfur nutrition could alter these metabolic systems which depend on UDP-glucose.

The data which are presented in Table 33 were collected during the course of an investigation of another type of stress, the stress of insecticide intoxication. These data show that if the diet is low in inorganic sulfate (0.0002%) but supplemented with 0.6% methionine, malathion intoxication depletes the body of sulfur, presumably the sulfur of mucopolysaccharides as evidenced by the decrease in hexosamine. However, if the level of inorganic sulfate is raised to 0.1%, then the depletion of body sulfur is not only halted but there is an apparent increase in mucopolysaccharide hexosamine with

Table 33. The effect of malathion on the total sulfur as sulfate and the hexosamine content of lungs in rats fed diets containing different levels of sulfate

Dietary sulfate	Total lung sulfur as sulfate		Total lung hexosamine	
	Without malathion	With malathion[1]	Without malathion	With malathion
	μg sulfate/mg lung		μg/mg lung	
0.0002%	29	25	2.6	2.2
0.1%	27	27	2.2	3.0
0.42%	24	27	2.2	2.4

[1]250 mg malathion/kg rat, dissolved in corn oil and administered by stomach tube.

malathion intoxication. When dietary inorganic sulfate is raised to the level of 0.42%, malathion intoxication appears to stimulate the formation of sulfated compounds in the lung even though there is a decrease in total hexosamine.

Previous data (Table 18), have shown that increased levels of dietary inorganic sulfate increase glycogen synthesis. The competition for UDP-glucose for glycogen synthesis and UDP-glucuronate synthesis for mucopolysaccharides would explain the decrease in mucopolysaccharide synthesis observed with higher levels of dietary sulfate (Table 33). Use of sulfate by malathion would remove this effect and allow the increased synthesis of mucopolysaccharides observed by the data shown in Table 33.

In addition to altering mucopolysaccharide metabolism, malathion has been shown by this laboratory to affect another ester sulfate-containing macromolecule: sulfolipid. When ester sulfate-containing macromolecules were extracted from cellular liver lipoprotein, a statistically significant increase was found in **in vivo** $^{35}SO_4^{=}$ incorporation into the sulfolipid fraction of malathion-stressed rats as compared with controls fed the diet containing 0.10% sulfate. It was hypothesized that this increase in specific activity is due to an attempt to replace sulfate mobilized from the livers of malathion-stressed rats. This hypothesis appears to be true since analysis revealed a lower sulfate content in cellular particulates of livers of rats treated with 250 mg malathion per kg of rats as compared with controls.

Since it is generally accepted that sulfolipid contains 1 mole of sulfate per mole of galactose (a), analysis of both sulfate and galactose was conducted in order to determine if the decrease in sulfate content mentioned earlier is due to a decrease in sulfate present in the galactose moiety of sulfolipid. As shown in Table 34, there is a

Table 34. Specific activity, mmole ratio and total galactose in the sulfolipid fraction of lipoprotein prepared from the cellular particulates of livers from control and malathion-treated rats[1]

Cellular particulates	Mg malathion/ kg of rat	Galactose		
		mg	cpm/mg x 10^{-2}	mmoles $SO_4^=$/ mmole
Nuclei	8	2.18	67	1.94
	150	1.67	121	0.92
Mitochondria	0	2.52	73	1.29
	250	2.04	42	0.42
Residue	0	1.84	36	1.66
	250	2.29	28	0.43

[1]Cellular particulates were extracted from 16 pooled livers from control rats and 20 pooled livers from malathion-treated rats fed a diet containing an intermediate level of sulfate (0.1%).

decrease in the mmoles $SO_4^=$ per mmole galactose when malathion is administered by stomach tube to rats. In order to test the hypothesis that the decrease in sulfate is a true phenomenon, the activity of arylsulfatase—an enzyme known to cleave sulfate off the sulfolipid molecule (b)—was determined in rats fed malathion and in control animals. In this method (72), p-nitrocatechol sulfate serves as the substrate for the enzyme arylsulfatase in a liver homogenate.

As shown in Table 35, the Ng sulfate-free nitrocatechol per mg nitrogen was significantly greater when rats were fed malathion as compared to controls. These data strongly indicate that the stress of malathion intoxication causes a mobilization of sulfate from sulfolipid. This alteration in sulfate metabolism has great implications when one considers the importance of sulfolipid to cellular membrane integrity and to the nervous system.

Table 35. The effect of malathion intoxication on liver arylsulfatase activity[1]

Mg malathion/kg of rat	μg nitrocatechol /mg N[2]
0	263 ± 17[3]
250	326 ± 20[3]

[1]Arylsulfatase activity was estimated in homogenates of three pooled livers of rats fed a diet containing an intermediate level of sulfate (0.10%).

[2]Mean of five replications $\pm$ SE.

[3]Difference is statistically significant ($P < 0.05$) by the method of unpaired comparisons (152).

The data shown in Table 34 illustrate the effect of the stress of avitaminosis E on sulfur metabolism. These data show a specific effect of avitaminosis E on sulfate incorporation by the lipoprotein and by the sulfolipid and mucopolysaccharide fractions associated with the lipoprotein. These data appear to show that it is the mucopolysaccharide fraction which contributes most to the overall decrease in specific activity shown by the lipoprotein from vitamin E-deficient rats. However, 3-5% of the total radioactivity was recovered in the mucopolysaccharide fraction and 12-14% in the sulfolipid fraction.

The decreased incorporation of $^{35}SO_4^{=}$ into the sulfolipid fraction of cellular lipoprotein may explain some of the symptoms of avitaminosis E. Brummer and Thomas (76) have shown the similarity of the cellular lipoproteins and the structural substance of a cell. Davidson and Gregson (77) found most brain sulfolipid to be associated with myelin and mitochondria and concluded that sulfolipids are true membrane lipids. Therefore, although a significant role for sulfolipids in membranes has not been determined, an alteration in sulfolipid metabolism as a result of the stress of avitaminosis E might explain the neurological symptoms of avitaminosis E.

The data presented in Table 36 may be used to further confirm a specific effect of avitaminosis E on the sulfolipids and mucopolysaccharides. It might be suggested that only those fractions which were actively metabolizing sulfate would be affected by avitaminosis E. However, the sulfate transfer fraction has a higher specific activity than any other fraction and loses it quicker. Yet the sulfate transfer fraction does not show a decreased incorporation of sulfate due to avitaminosis E. Therefore, these data show that of the compounds studied, avitaminosis E specifically affects only the sulfation of (liver) sulfolipids and mucopolysaccharides.

The data which have been presented provide strong evidence that inorganic sulfate and/or the ratio of neutral sulfur to sulfate, and/or the role of cysteine and/or methionine are involved in various metabolic processes in at least one monogastric animal, the rat. These data have also shown that with a decreased intake of inorganic sulfate there is an increased requirement for neutral sulfur to supply the total sulfate need.

However, most of the publicity concerning sulfur is directed towards its polluting qualities. Sulfur pollution has been an environmental concern for more than 20 years. In certain areas, the sulfur dioxide (SO_2) content of the air has been high enough to damage vegetation and presumably injure human health. These hazardous effects have led to strict pollution legislation restricting the amount of sulfur in various forms that may be emitted into the atmosphere. Alterations in one component of the sulfur cycle (Figure 9) with little attention to the other components could be disastrous. The

Table 36. Effect of A-vitaminosis E on the uptake of $^{35}SO_4^{=}$ by liver cell lipoproteins

Lipoprotein component	Specific activity of component			
	8 hour[1]		24 hour	
	Sufficient E	Deficient E	Sufficient E	Deficient E
Complete lipoprotein counts/min/mg	5.7	4.7	3.9	3.4
Lipoprotein fractions lipid fractions counts/min/m mole $SO_4^{=} \times 10^{-4}$	5.4	3.9	5.0	3.3
Mucopolysaccharide fraction counts/min/mg	42	18	33	11
Nucleotide fractions total counts/min $\times 10^{-2}$	12	16	21	19
Sulfate transfer fraction counts/min/mg	53	65	19	17
Residue counts/min/mg	3.7	3.6	4.2	3.9

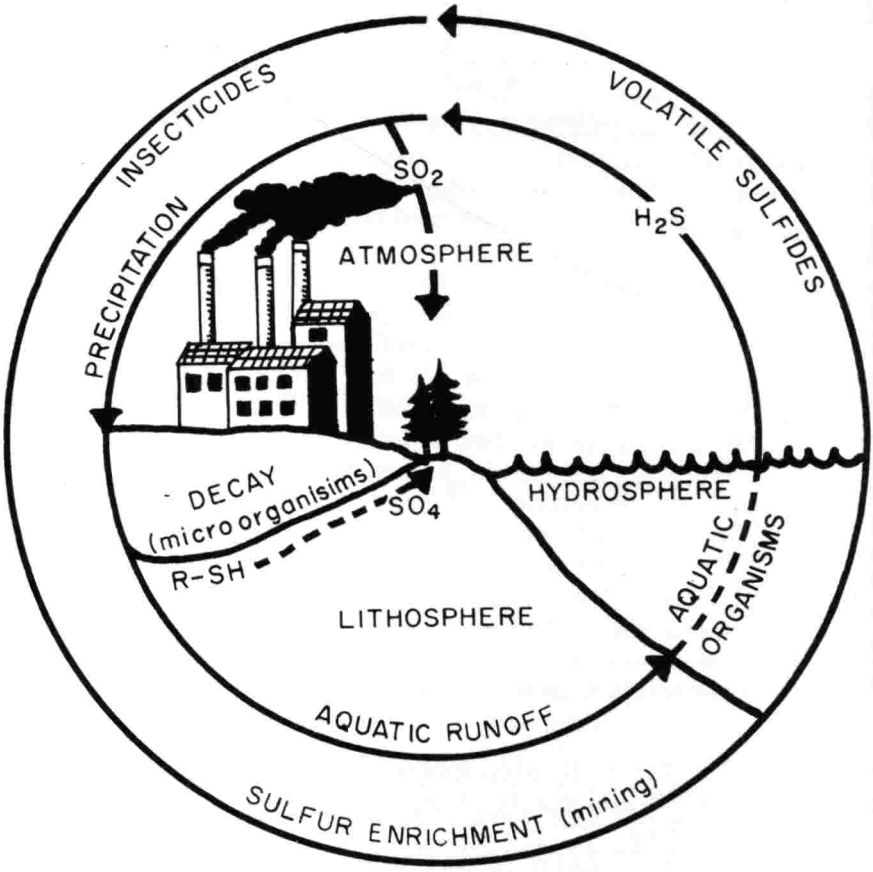
[1]Rats sacrificed 8 and 24 hours after the pulse dose of $^{35}SO_4^{=}$.

reduction of sulfur in the atmosphere could result in not only a reduction of quantity and quality of crops, but also a possible reduction in human well-being.

There are four major factors to be considered in evaluating sulfur balance between air pollution and supply to the land (78). First, there are large areas of the world where sulfur is deficient, including parts of Central Africa, much of the Midwest and West U.S.A., and small parts of Great Britain and Central Ireland (78-80). Second, the rising cost of labor is leading farmers to use high analysis fertilizers which contain little sulfur. Third, there are areas where sulfur pollution is excessive. And fourth, if fossil fuels were available and allowable, a general increasing output of SO_2 from combustion would be predicted (78, 79).

Using sulfur in the atmosphere for vegetational use could redistribute the sulfur locked in fossil fuels. However, since concentrations of SO_2 are high near the point of discharge, the tolerable concentrations must be determined. At present it appears that concentrations above 400 ug/m^3 should be restricted to limited duration in order to avoid harm to vegetation. However, man and other animals appear to be far more tolerable than vegetation to high levels of SO_2

Figure 9. The sulfur cycle.



(81). This level of 400 ug/m^3 is well above the annual arithmetic mean allowable of 60 ug/m^3 set by the Environmental Protection Agency. In fact, a maximum 24-hour concentration of 260 ug/m^3 is not to occur more than one time a year. These pollution abatement regulations which are world wide have resulted in increased use of low sulfur fuels, scrubbers, and precipitators and have created the necessity of adding sulfur to fertilizers.

Therefore, in view of the data presented in this bulletin with respect to the importance of sulfur nutrition to mammals, it is suggested that because the sulfur cycle is so important, the sulfur emission standards should be revaluated and/or consideration should be given to the use of sulfur in fertilizers. These measures appear to be necessary to insure not only the health of man but the availability of mans' food supply.

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THE UNIVERSITY OF TENNESSEE
AGRICULTURAL EXPERIMENT STATION
KNOXVILLE, TENNESSEE 37901

(2M/3-78)